To the Graduate Council:

I am submitting herewith a thesis written by Steven Allen Sanders entitled "Immediate-early Polypeptides of Herpes Simplex Virus Type 2 Infected Cells." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Richard J. Courtney,
Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

IMMEDIATE-EARLY POLYPEPTIDES OF HERPES SIMPLEX VIRUS TYPE 2 INFECTED CELLS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Steven Allen Sanders

August 1981

3062942

DEDICATION

For all the ladies in my life -
My mother, daughters, and my wife;
In all the earth, sea and sky,
Only these provide the reasons why.

ACKNOWLEDGEMENTS

I am very fortunate to have been surrounded by friends and loved ones who offered nothing but encouragement and support through all the challenges offered by this endeavor. My laboratory comrads Liz Wenske and Mike Bratton will always be considered veterans of hard fought and long suffered battles. Our many late-night introspective disquistions will always be appreciated as instrumental events in the culmination of this effort. The genuine love and friendship offered to all by Ms. Karen Moreau is an invaluable commodity. An irreplaceable and irrevocable feeling of love and welcome will be maintained for her in the hearts of all fortunate enough to know her. And to my fellow students, Ms. Jan Lewis and Dr. Richard Eberle, a special thanks for creating an atmosphere conducive to scientific thought and investigation; and also for helping to welcome me into a highly respected and productive laboratory.

I greatly appreciate the interest, understanding, and support shown me by the faculty members of my research committee: Professors Richard J. Courtney, Carl J. Wust, and Arthur Brown.

ABSTRACT

Herpes simplex virus type 2 infected cells produce virus-specific polypeptides in three coordinately controlled kinetic classes. The immediate early (α) class of infected cell polypeptides is thought to be functionally important to the HSV-2 polypeptide synthesis control mechanism. The α polypeptides (ICP4, ICP0, and ICP27) have been purified by high resolution preparative polyacrylamide gel electrophoresis containing sodium dodecyl sulfate. Monospecific antisera have been produced against these highly purified proteins and used as immunologic probes to characterize their intracellular expression in HSV-2 infected cells. Antisera produced against two of the α polypeptides (ICP4 and ICP27) have been shown by indirect immunofluorescence to react equally with herpes simplex virus type-1 (HSV-1) and HSV-2 infected cells. The antiserum produced against the ICP0 α polypeptide, however, reacted only with HSV-2 infected cells. The times of appearance and intracellular migration of the α polypeptides have also been examined by immunofluorescence, and it was found that: 1) ICP4 migrates to the nucleus of HSV-2 infected cells where its presence is detectable through 10 hours post infection (hrs pi); 2) ICP0 migrates to the nucleus of HSV-2

infected cells but is only detectable during the first six hours of infection; and 3) ICP27 is expressed in the cytoplasm of HSV-2 infected cells from three through ten hrs pi. The times of appearance and intracellular migration of the α polypeptides have also been examined in HSV-2 infected cells cultured in the presence of amino acid analogues (canavanine and azetidine) which are known to alter the control mechanism of HSV-2 polypeptide synthesis. The times of appearance and intracellular location of HSV-2 infected cell polypeptides cultured in the presence or absence of amino acid analogues have been used to propose a model of HSV-2 polypeptide synthesis control which implicates the α polypeptides in the induction or maintenance of the HSV-2 lytic cycle and the latent state.

TABLE OF CONTENTS

CHAPTER PAGE

LIST OF FIGURES

FIGURE PAGE

FIGURE PAGE

INTRODUCTION

Herpes simplex viruses are associated with a variety of medically important diseases ranging from recurrent oral and genital lesions to a possible involvement with cancer of the lip and cervix. Nahmias and Roizman (1973) have shown that 70 to 95% of the population shows seroepidemiological evidence of past HSV-1 infection by late childhood, whereas HSV-2 is acquired by venereal transmission later in life (Roizman and Kieff, 1975). Definitive serological evidence of HSV-1 or HSV-2 infection is difficult to demonstrate due to the cross-reactivity of the two virus types, but Rawls, et al. (1970) developed a procedure to distinguish between them by use of a II/I index based on the ratio of titers of specific neutralizing antibodies to HSV-2 or HSV-1. The HSV-1 virus, once acquired, is thought to be particularly prone to harbourage in sensory neurons (Klein, 1976) and to establish latency in the sensory ganglion near the site of infection (trigeminal) (Nahmias and Roizman, 1973). This virus has the unfortunate capability of traversing the nerve root and establishing a usually fatal viral encephalitis. Ojeda (1980) has also demonstrated that alternate routes of encephalitic infection exist and has anatomically associated these

routes with several cerebral sites from which the virus has been isolated. The HSV-2 virus is also capable of establishing a latent infection which is thought to be mediated through the relationship of the virus with the lumbo-sacral ganglion; a situation analogous to the HSV-1 trigeminal ganglion relationship.

Much evidence has accumulated relating the HSV-2 infection with cervical carcinoma (Naib, et al., 1966; Rawls, et al., 1968). Aurelian, et al. (1971, 1973) and Hollinshead, et al. (1973) have isolated and identified specific antigens from HSV-2 infected cells that react with antisera from cervical carcinoma patients. The antigen isolated by the Aurelian group (AG-4) is an early antigen, obtained from cells harvested 4 hours post infection, with an apparent molecular weight of 161,000. This antigen was detected with a complement fixation assay using serum obtained from patients with cervical carcinoma and has been demonstrated to be analogous to an early, non-structural polypeptide found in HSV-infected cells. The antigen isolated by the Hollinshead group (HSV-TAA), also detected by complement fixation, was obtained from HSV-2 infected cells harvested at 24 hours post infection (hrs pi). Notter and Docherty (1976) compared antisera obtained from cervical carcinoma patients with

that obtained from normal controls and found a significant reaction to the AG-4 and HSV-TAA antigens with the cervical carcinoma sera.

Evidence supporting the herpesvirus hypothesis in the epidemiology of cervical cancer gained much impetus with the discovery by Duff and Rapp (1971) of the ability of HSV-1 and HSV-2 to transform mammalian cells in vitro. This discovery was corroborated by many laboratories (Munyon, et al., 1971; Davidson, et al., 1973; Kucera, et al., 1975; Kucera and Gudson, 1976) as was the actual oncogenicity of the HSV-transformed cells (Duff and Rapp, 1973; Kucera, et al., 1977; Macnab, 1974; Li, 1975). Transformed cells have been examined by several laboratories using nucleic acid hybridization techniques (Frenkel, et al., 1972; 1976; Collard, et al., 1973; Minson, 1976) and several HSV-specific nucleotide sequences have been found. HSV genome sequences lying between map units 0.2 and 0.4 are commonly found in HSV-transformed cells with some transformed cells containing variable amounts of HSV DNA outside of these co-ordinates (Minson, 1979; Leiden, 1980). Camacho and Spear (1978) have succeeded in transforming hamster embryo fibroblasts with a restriction endonuclease fragment located from 0.3 to 0.45 map units on the HSV-1 genome. In addition

to HSV DNA, HSV-1-transformed cells have been found to contain HSV-specific antigens (Duff and Rapp, 1971; 1973; Camacho and Spear, 1978; Lewis and Courtney, 1981). In addition to the aforementioned HSV-specific antigens detected by using sera from carcinoma patients, several other antigens have been found associated with HSV-transformed cells. Of particular interest is an early, non-structural HSV-2 polypeptide (VP143, 143,000 m.w.) which has been reported as an internal antigen in HSV-transformed cells (Kimura, 1975; Melnick, et al., 1976; Flannery, et al., 1977; Lewis and Courtney, 1981).

Early antigens produced in virus-infected cells are very interesting to the molecular biologist because of the pivotal role they play in the virus replication scheme and also because of their purported involvement in cell transformation. T-Ag found in the SV-40 and polyomavirus systems is the best example of the involvement of an early polypeptide in the DNA tumor virus transformation process and indeed makes the search for early-polypeptide involvement in the HSV-transformation process inviting.

The immediate-early polypeptides produced in HSV-2 infected cells are important because of their pivotal role in the cascade regulation of the HSV-2 infectious cycle. These polypeptides are also

important due to their possible involvement with cell transformation as well as the induction or maintenance of the latent state. The first step toward defining the role of these polypeptides in the infectious process is to gain a thorough understanding of the functional importance of the immediate-early polypeptides in the lytic cycle of HSV-2. Antiserum produced against one of the immediate-early polypeptides (ICP4) isolated from HSV-1 infected cells has been used by our laboratory to gain an understanding of the functional role of this polypeptide in the lytic cycle (Courtney and Benyesh-Melnick, 1974). Anti-ICP4 antiserum produced against the HSV-1 antigen has also been used to detect the polypeptide in the trigeminal ganglion cells excised from rabbits latently infected with HSV-1 (Green, et al., 1981). The availability of monospecific antisera to the immediate-early polypeptides produced in HSV-2 infected cells would greatly facilitate the development of an understanding of the functional role of these polypeptides in the infectious cycle. It would also provide a means to identify the potential role these HSV-2 polypeptides play in the establishment and/or maintenance of latency and transformation.

The specific aims of this research project include: 1) the production of monospecific antisera

to highly purified polypeptides of the entire immediate-early (α) class of HSV-2 infected cell polypeptides; 2) the determination of the cross-reactivity of the immediate-early polypeptides produced in HSV-1 or HSV-2 infected cells; 3) the determination of the times of expression and intra-cellular location of the antigen produced in lytically infected cells; and 4) an examination of the effects of amino acid analogues on the expression of the polypeptides produced in cells cultured in their presence.

CHAPTER I

LITERATURE REVIEW

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), members of the family Herpetoviridae, are large DNA-containing enveloped viruses which infect primarily vertebrate hosts. The capsid, which is an icosohedron comprised of 162 capsomeres with a diameter of approximately 100nm, contains a core consisting of protein and DNA arranged in a toroidal structure (Furlong, et al., 1972). The DNA is double stranded and linear with a molecular weight (mw) of approximately 100 X 10^6 daltons (Roizman, 1979). There is a coding capacity available for approximately 75 proteins, of which 24 have been identified as structural (Spear and Roizman, 1972) and 16 as non-structural, with all the other identified HSV-infected cell polypeptides (ICP's) remaining unclassified (Honess and Roizman, 1973). The virus enters the cell either by viropexis (Holmes and Watson, 1961; Dales and Silverberg, 1969) or, more probably, by fusion of the viral envelope with the cell membrane (Morgan, et al., 1968; Hummeler, et al., 1969; Abodeely, 1970). Virus multiplication begins in the nucleus with the production and assembly of virus nucleocapsids (Andrewes, et al., 1978). The envelope is acquired as the capsid passes

through the inner lamella of the nuclear membrane (Morgan, et al., 1954) and the mature infectious virus (Watson, et al., 1963) appears to traverse the cytoplasm via membranous channels and to be released from cells by budding.

The classification of the herpes simplex viruses into type 1 or type 2 was originally based upon the anatomic site of isolation of the particular strain, with viruses isolated from oral and opthalmic lesions designated HSV-1 and viruses isolated from genital lesions designated HSV-2. This criterion for classification has given way to a more rigorous biochemical definition which has shown that both HSV-1 and HSV-2 can be isolated from either anatomical site. The currently accepted criteria for classification as type 1 or type 2 HSV include polyacrylamide gel analysis of restriction endonuclease cleaved HSV-DNA (Skare, et al., 1975; Summers, 1980), polyacrylamide gel electrophoresis (PAGE) migration patterns of certain HSV-specific polypeptides (Cassai, et al., 1975; Courtney, 1976; Pereira, et al., 1976; Killington, et al., 1977; Bookout and Levy, 1980), and/or a positive reaction in immunodiagnostic tests using monospecific antisera produced against type-specific HSV antigens (Eberle and Courtney, 1981). The two types of viruses are genetically very closely related, having approximately 50% of their

genomic sequences in common (Kieff, et al., 1972). Many of the virus encoded polypeptides, both structural and non-structural, are similar in molecular weight, kinetics of synthesis, modification, intracellular localization, and apparent function (Honess and Roizman, 1974; Powell and Courtney, 1975; Powell and Purifoy, 1977). Immunoprecipitation tests using antisera prepared either against extracts of HSV-infected cells or in direct response to a primary infection show the vast majority of the HSV-specific polypeptides to be cross-reactive (type-common) (Honess and Watson, 1977). The search for type-specific antigens began with neutralization studies using antisera cross adsorbed with the two strains of virus (Sim and Watson, 1973; Powell, et al., 1974) and have continued using intertypic viral recombinants (Halliburton, et al., 1977). These studies have defined at least four type-1 specific neutralization sites and three type-2 specific neutralization sites. Immunofluorescence has been used to demonstrate that at least one non-structural polypeptide (ICP4) is type-1 specific (Courtney and Benyesh-Melnick, 1974).

Infection of permissive cell lines with HSV has a profound effect on cell metabolism and morphology. Lytically infected cells are seen to have a very rapid termination of protein synthesis and also a cessation

of DNA synthesis (Hamada and Kaplan, 1965; Sydiskis and Roizman, 1967; 1968; Honess and Roizman, 1973; Fenwick, et al., 1979). Polyribosomes normally associated with cellular mRNA are seen to rapidly dissociate (Silverstein and Engelhardt, 1979), with the concomitant cessation of protein synthesis and the ensuing degradation of the naked cellular mRNA. The dissociation is independant of viral protein synthesis since cells enucleated after HSV-infection or infected in the presence of actinomycin D are still seen to exhibit the inhibitory effect (Fenwick and Walker, 1978). HSV-1 affects the inhibition of cellular protein synthesis by 1.5 to 2.0 hours post infection (hrs pi) whereas the HSV-2 host cell inhibition occurs a little earlier in the infectious cycle, from 0.5 to 1.0 hrs pi (Powell and Courtney, 1975; Pereira, et al., 1977; Morse, et al., 1978; Fenwick, et al., 1979). Host cell RNA synthesis is not inhibited as is the host cell DNA and protein synthesis, but there is very little ribosomal RNA made, and the mRNA which is made is not efficient at directing protein synthesis (Wagner and Roizman, 1969; Jacquemont and Roizman, 1975). This finding is particularly interesting in light of the similarities between the host cell RNA polymerase which is primarily responsible for transcription of ribosomal RNA (RNA polymerase II)

and the polymerase responsible for transcription of HSV mRNA. Costanzo (1977) found that HSV-specific transcription is sensitive to the effects of α-amanitin (as is the host cell RNA polymerase II) when grown in cells sensitive to the drug, but is resistant when grown in α-amanitin-resistant cells. There has also been evidence found (Leung, 1980) describing a functional relationship between HSV immediate-early polypeptides and an altered affinity of the RNA polymerase II molecule favoring transcription of HSV-specific genes.

Polypeptide Synthesis in HSV-1-infected Cells

The first thorough examinations of herpes simplex virus polypeptides using the high resolution technique of polyacrylamide gel electrophoresis (PAGE) were done by Spear and Roizman (1972) on purified enveloped virions of HSV-1 and by Gibson and Roizman (1972) on purified nucleocapsid proteins. These studies identified the major capsid protein (ICP5), several minor virus structural proteins, and three major envelope glycoprotein regions (including the VP123 major glycoprotein region). Early analyses of polypeptides produced in HSV-infected cells demonstrated a very early initiation of virus specific polypeptide translation and migration to the nucleus (Spear and Roizman, 1968), a dependence on arginine

for the intracellular migration of HSV-specific polypeptides (Courtney, et al., 1970; 1971), and analysis of the amino acid content of HSV-infected cell polypeptides (Kaplan, et al., 1970). Honess and Roizman (1973) examined the kinetics of HSV-specific protein synthesis and the relative molar ratio of structural to non-structural polypeptides. Though there was no remarkable difference in the molar ratios of structural and non-structural proteins found, there was an obvious temporal regulation of protein synthesis and the distribution of structural and non-structural polypeptides among the regulatory patterns seemed specific rather than random.

A further evaluation of the kinetics of protein synthesis in HSV-1 infected cells provided a great deal of insight into the regulatory control mechanisms of HSV protein production. The polypeptides were found to be produced in a coordinately controlled and sequentially ordered type of cascade regulation wherein one group of HSV-specific polypeptides controls (and, in turn, is controlled by) the synthesis of the next group of polypeptides. Three such coordinately controlled groups on HSV-infected cell polypeptides were found (designated α, β, and γ) (Honess and Roizman, 1974) and have been further divided into subgroups based upon minor differences in the kinetics

of synthesis of polypeptides within each group and on the choice of metabolic inhibitor used to dissect the system (Honess and Roizman, 1974; 1975; Pereira, et al., 1977).

Upon infection of permissive cells with HSV-1, the synthesis of the first group of virus-specific polypeptides, the immediate-early or α polypeptides, begins immediately and reaches a peak production rate at 3 to 4 hrs pi (Honess and Roizman, 1974). These non-structural polypeptides are produced in the absence of previous viral protein synthesis and are the first polypeptides produced in infected cells after the removal of a cycloheximide or puromycin block. The presence of α polypeptides allows the initiation of the next group of proteins, the early or β class polypeptides (β polypeptides), which reaches its peak rate of synthesis at 5 to 7 hrs pi (Honess and Roizman, 1975). This group of polypeptides does require prior HSV-specific protein synthesis, specifically, functional α polypeptides (Honess and Roizman, 1975; Pereira, et al., 1977). The β polypeptides are comprised of both structural and non-structural proteins, including the viral thymidine kinase and DNA polymerase (Powell and Purifoy, 1977; Purifoy and Powell, 1977). Regulatory functions of the β polypeptides include the inhibition of α polypeptide synthesis and the initiation

of late or γ class polypeptide synthesis. The inhibitory function of the β polypeptide class was demonstrated by the addition of L-canavanine (an arginine analogue) during the time of synthesis of the β polypeptides. Polypeptides synthesized in the presence of canavanine presumably have an altered tertiary structure and would therefore exhibit altered functional characteristics. Because of this effect, amino acid analogues have been used at the time of infection to hinder the regulatory function of the HSV-specific polypeptides and thus allow only the production of the α polypeptide class of proteins. When canavanine was added prior to 1.5 hrs pi (Honess and Roizman, 1975) only one β polypeptide (ICP6) was produced, and the synthesis of the α polypeptides was seen to continue unabated until more than 18 hrs pi. Addition of canavanine at 4 hrs pi was seen to allow the normal termination of α polypeptide synthesis, but did result in a reduced rate of synthesis of γ polypeptides. The γ class polypeptides are all thought to be structural components of the virion itself. The mechanisms controlling the α, β, γ shifts are not well understood (Fenwick and Walker, 1979; Leung, 1980).

Several laboratories have undertaken studies of the HSV-specific RNA produced in infected cells to help elucidate the control mechanisms of cascade

regulation (Subak-Sharpe and Hay, 1965; Frenkel and Roizman, 1972; Wagner, et al., 1972; Swanstrom, et al., 1975; Preston, 1979). It was found that both strands of HSV DNA are transcribed into messenger RNA (BenZeev and Becker, 1975; Kozak and Roizman, 1975) and that the transcription occurs in a regulatory fashion analogous to HSV-polypeptide production (Jones and Roizman, 1979; Holland, et al., 1979). Analysis of cytoplasmic RNA's by both in vitro translation and by RNA-DNA hybridization techniques using restriction endonuclease cleaved fragments of DNA gave results consistent with previous knowledge about the cascade regulation of protein synthesis. Talley-Brown and Millette (1979) demonstrated that cytoplasmic RNA isolated in the presence of cycloheximide could effectively direct only α polypeptide synthesis in vitro. Similar studies were done using the RNA-DNA hybridization technique of Southern to define the physical map location of α polypeptide specific RNA messages (Anderson, et al., 1979; Watson, et al., 1979).

Immediate-early Polypeptides of HSV-1-infected Cells

The immediate-early (α polypeptide) class of HSV-1-infected cell polypeptides is comprised of a small number of polypeptides ranging in molecular weight from 60,000 daltons to 175,000 daltons (Honess and Roizman, 1974), all of which are phosphorylated.

The actual number of polypeptides in this group has been debated since its inception (Kozak and Roizman, 1974; Honess and Roizman, 1975). The four polypeptides which are commonly accepted as members of the group are ICP4, ICP0, ICP22, and ICP27 (see Figure 1 for nomenclature and approximate molecular weights).

ICP6 and ICP16 are β polypeptides made very early in infection and classified by some investigators as members of the α class polypeptides because of their kinetics of synthesis using either cycloheximide (ICP6) or azetidine (ICP16) (Preston, et al., 1978). One small polypeptide (12,500 daltons), which migrates at or near the dye band in 7% PAGE analysis, has also been assigned to this group and there is evidence from RNA-DNA hybridization experiments which supports this designation (Easton and Clements, 1980). The apparent confusion as to which polypeptides belong to the α group arise from 1) the variable and often early onset of β polypeptide synthesis; 2) the type of metabolic inhibitor used to interrupt protein synthesis in order to dissect the events of cascade regulation; and 3) the type (HSV-1 or HSV-2) of virus used for studying the synthesis and properties of this kinetic class of polypeptides.

ICP4. The most intensely studied of the α polypeptides is ICP4, the largest of the four at 175,000 daltons (Courtney and Benyesh-Melnick, 1974; Courtney,

Figure 1. Identification of certain HSV infected cell polypeptides. Autoradiograms of detergent extracts of HSV-1 infected cells (EXT) radioactively labeled with ^{35}S-methionine from 3-6 hours post infection (hrs pi) or cells infected with HSV-2 and labeled from 2-4 hours with ^{35}S-methionine either in the presence of canavanine (CAN) or in the absence of canavanine (EXT). The nomenclatures of Roizman and co-workers (a and c) (Honess and Roizman, 1974) and Courtney (b and d) (Courtney, 1976) are presented. The molecular weights of the infected cell polypeptides can be determined from the nomenclature of Courtney, which is presented as the molecular weight of a viral polypeptide times 10^{-3}.

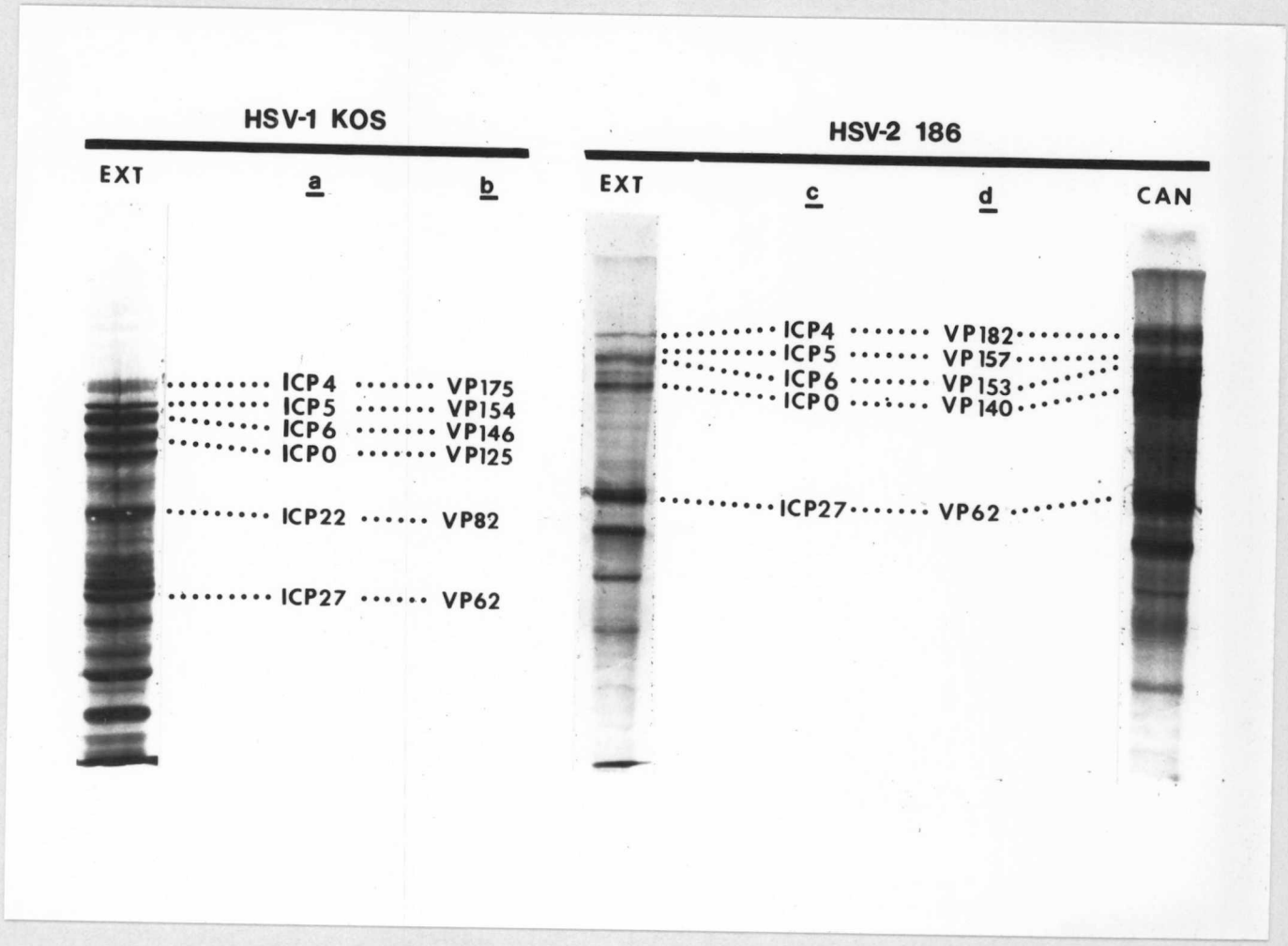
HSV-1 KOS
EXT
a
b
ICP4 VP175
ICP5 VP154
ICP6 VP146
ICP0 VP125
ICP22 VP82
ICP27 VP62
HSV-2 186
EXT
c
d
CAN
ICP4 VP182
ICP5 VP157
ICP6 VP153
ICP0 VP140
ICP27 VP62

Figure 1.

et al., 1976). This polypetide apparently plays a dominant role in the cascade regulation of protein synthesis in HSV-1 infected cells because it is the first polypeptide produced (Honess and Roizman, 1974; Courtney and Benyesh-Melnick, 1974) and because it is found to be tightly associated with HSV-infected cell nuclei and DNA (Powell and Purifoy, 1976; Fenwick, et al., 1978; Hay and Hay, 1980). The polypeptide is translated from immediate-early mRNA (Holland, et al., 1979; Easton and Clements, 1980) in the cytoplasm and is then rapidly transported to the nucleus, where it is assumed to participate in a regulatory function. The transport of this polypeptide is seen to occur with the concomitant modification of the original form (ICP4a) to at least two forms of higher apparent molecular weight (Honess and Roizman, 1975; Courtney and Powell, 1975). These forms are all phosphorylated and are designated ICP4a, ICP4b, and ICP4c. Cells fractionated into cytoplasmic and nuclear components show the ICP4c form associated with the nuclear fractions only, whereas the ICP4a and ICP4b components are also contained in the cytoplasm (Pereira, et al., 1977; Wilcox, et al., 1980).

Studies aimed at defining the function of ICP4 have been conducted using cells enucleated after the transcription of α polypeptide messages and also with the cells infected with ts mutants with a lesion in the gene

coding for ICP4. Enucleated cells are shown to produce all three forms of ICP4, all of which appear to have the same electrophoretic mobility as those produced in nucleated cells (Fenwick and Roizman, 1977). Synthesis of ICP4 is prolonged if the cells are enucleated prior to the transcription of β or γ polypeptides but is terminated if enucleation occurs after the β and γ transcripts are made and transported to the cytoplasm. The gene encoding ICP4 is located in the reiterated terminal segments of the S fragment of the genome (Frenkel, et al., 1975; 1976; Preston, et al., 1978) (see HSV genetic map, Figure 2) and has been shown to be diploid by using intertypic recombinants (Knipe, et al., 1978). Both copies of the gene are transcribed and functional polypeptides are made from each (Knipe, et al., 1978; Morse, et al., 1978). ts mutants with a lesion in the ICP4 gene (tsB2 complementation group, Schaffer, et al., 1973) are DNA^- and are defective in initiating the synthesis of proteins belonging to subsequent kinetic classes (Courtney, et al., 1976; Schaffer, et al., 1976). Cells infected at the non-permissive temperature (39°C) produce a protein which is not processed to the higher molecular weight (ICP4c) species (Courtney, et al., 1976), and which does not segregate into cytoplasmic and nuclear fractions as does the native protein. Courtney and Benyesh-Melnick (1974) showed using immunofluorescence

Figure 2. Herpes simplex virus genome. Graphic representation of the physical map location of the coding region of certain HSV-specific polypeptides of the immediate early, early, and late kinetic classes (Morse, et al., 1978; Preston, et al., 1978). The following abbreviations were used to represent the portions of the HSV genome: L, large component; S, small component; IR_L, internal repeat, long component; TR_L, terminal repeat, long component; IR_S, internal repeat, short component; TR_S, terminal repeat, short component; U_L, unique sequences, long component; U_S, unique sequences, short component.

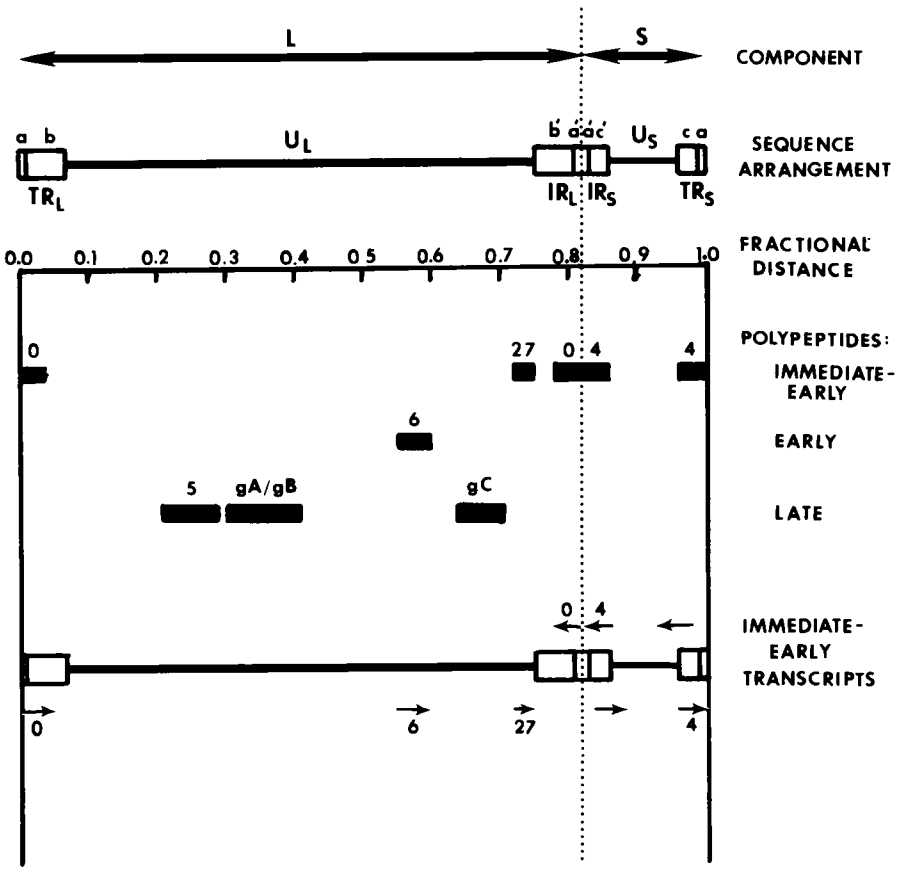
L
S
COMPONENT
a b
U_L
b' a' a c' U_S c a
SEQUENCE ARRANGEMENT
TR_L
IR_L IR_S
TR_S
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
FRACTIONAL DISTANCE
POLYPEPTIDES:
0
27 0 4
4
IMMEDIATE-EARLY
6
EARLY
5
gA/gB
gC
LATE
0 4
IMMEDIATE-EARLY TRANSCRIPTS
0
6
27
4

Figure 2.

that ICP4 accumulated in the nucleus of infected cells at permissive temperatures but was seen dispersed throughout the cytoplasm and nucleus at the non-permissive temperature. Preston (1979) showed that tsK mutants (also defective in the ICP4 gene) had the same characteristics as the tsB mutants described above, and in addition, showed that the ts-polypeptide was not phosphorylated at the non-permissive temperature.

Wilcox, et al. (1980) have performed the most extensive examination of HSV-infected cell phosphoproteins and have reported that there are at least two mechanisms of phosphorylation involved: one in which the phosphate is permanently bound and one in which the phosphate is seen to cycle on and off the protein moeity. ICP4a and ICP4c are phosphorylated by the cyclic mechanism, as is shown by the continued ability to phosphorylate these species many hours after the termination of their translation, whereas the ICP4b species is stabily phosphorylated following translation. This modification is not associated with either the intra-cellular compartmentalization or the increase in apparent molecular weight but apparently is involved with the polypeptide's DNA binding affinity. This finding led the investigators (Wilcox, et al., 1980) to hypothesize that ICP4c (and perhaps also ICP4a) is utilized by the virion in a cyclic manner by repeatedly altering the molecule's specific DNA-binding affinity in

order to alter its regulatory function. The labeling of infected cell polypeptides in the presence of canavanine showed that even the canavanine-analogue of the polypeptide is extensively phosphorylated, an indication that since the phosphorylation occurred in the absence of β and γ polypeptide synthesis, it is mediated either by a cellular protein kinase or else by the kinase activity of one of the α polypeptides themselves (Pereira, et al., 1977).

ICP0. ICP0 is a 125,000 dalton protein (Courtney, et al., 1976) which is made according to all the specifications for classification as an α polypeptide. This particular protein has the dubious distinction of being the only polypeptide known to be overlooked when the original infected cell polypeptides were enumerated. The reason for the oversight is because of the comigration of this protein with ICP8 (Honess and Roizman, 1974), a β polypeptide whose synthesis begins relatively early in infection and continues throughout the infectious cycle. ICP0 is a relatively unstable polypeptide. When pulse labeled from 2 to 4 hours pi in HSV-1 infected cells it can be shown to be found in the nucleus and cytoplasm (Pereira, et al., 1977) but is thought to deteriorate within a few hours of production (Wilcox, et al., 1980). This rapid deterioration is in sharp contrast to all the other α polypeptides, which are shown to be retained in stabile form through 24 hrs pi. In some strains of HSV, ICP0 can be seen to have a slightly

different migration rate from ICP8 and is thus easier to identify (Honess and Roizman, 1974; Fenwick, et al., 1980). The gene coding for ICP0 has also been shown to be diploid (as is the gene for ICP4), being mapped in the reiterated sequences of the L segment of the genome (Preston, et al., 1978). Because of the early synthesis and short half-life of the polypeptide, it must have a very early function in infection (Spear and Roizman, 1980).

ICP22. ICP22 is an 82,000 dalton polypeptide which is known to be phosphorylated (Pereira, et al., 1977) and modified in a step-wise manner to a slower migrating form. The map location for the gene encoding ICP22 is not known. The polypeptide is made immediately after the removal of a cycloheximide block (Kozak and Roizman, 1974) and its synthesis is maintained when the HSV-infected cells are cultured in the presence of azetidine (Fenwick, et al., 1980), as are all the α polypeptides. The kinetics of synthesis of ICP22 differs from that of the other α polypeptides, however, in that its synthesis is not terminated as rapidly by the appearance of β polypeptides (Fenwick, et al., 1980). ICP22 is isolated in the nuclear fraction of homogenized cells (Fenwick, et al., 1978), but this association has not been well characterized.

The identification of ICP22 as an HSV-specific α polypeptide has been shrouded with much controversy. After

originally being described as an early protein of HSV-infected cells (Honess and Roizman, 1973; Kozak and Roizman, 1974), its HSV-specificity was questioned because of the discovery of an uninfected cell polypeptide of similar molecular weight whose synthesis was stimulated by growth in the presence of canavanine or growth at 38.5°C (Honess and Roizman, 1975). Studies conducted by Fenwick, et al. (1980) to settle this confusion have shown that ICP22 differs in molecular weight from the heat-shock proteins of Vero cells grown at elevated temperatures. In addition, ICP22 was shown to be phosphorylated and to accumulate in the nucleus, whereas the heat-shock proteins are non-phosphorylated and shown to remain in the cytoplasm (Fenwick, et al., 1980). The conclusions drawn were that ICP22 is indeed a HSV-specific polypeptide and that it belongs in the α class of HSV-infected cell polypeptides.

ICP27. The fourth of the HSV-1 infected cell α polypeptides (ICP27) is a phosphoprotein with a molecular weight of 62,000 (Courtney, et al., 1975; Honess and Roizman, 1974; Fenwick, et al., 1980). This polypeptide is found in both nuclear and cytoplasmic cell fractions and has not been shown to have a detectable change in molecular weight (Pereira, et al., 1977). When produced in the presence of canavanine, ICP27 remains entirely in the cytoplasm (Pereira, et al., 1977).

ICP27 has been shown by RNA-DNA hybridization studies using endonuclease cleaved DNA to map between 0.741 and 0.752 map units on the HSV genome (Watson, et al., 1979; Easton and Clements, 1980). This location is in the unique sequences found just inside the internal repeat of the long segment (IR_L) of the genome and thus would be the only immediate-early polypeptide found mapping outside of the reiterated sequences. This map location is in slight variance with that found by other workers (Morse, et al., 1978; Preston, et al., 1978) who reported that ICP27 mapped just inside the IR_L segment with a slight extension of the gene into the internal reiterated sequence itself.

Immediate-early Polypeptide Synthesis in HSV-2 Infected Cells

The molecular biology of herpes simplex virus type 2 (HSV-2) has not been studied to the extent of the type 1 virus. The two viruses are thought to share about a 50% sequence homology (Ludwig, et al., 1972; Sugino and Kingsbury, 1976) and readily undergo intertypic recombination (Timbury and Subak-Sharp, 1973; Fenwick, et al., 1979; Ruyechan, et al., 1979). The viruses are also known to undergo itertypic complementation and marker rescue (Esparza, et al., 1976; Chartrand, et al., 1979). The HSV-1 X HSV-2 intertypic recombinants have been used

fruitfully to study the phenomena of host cell inhibition (Halliburton, et al., 1976), the expression of immediate-early polypeptides (Preston, et al., 1978), and to map various functions or gene locations (Morse, et al., 1978; Halliburton, 1980). The host-cell shut-off exhibited by the herpes simplex viruses is much more dramatic in type-2 infected cells than in type-1 infected cells. Fenwick, et al. (1979) studied this inhibition using HSV-1 X HSV-2 intertypic recombinants and mapped the function between 0.52 and 0.59 map units of the HSV genome. There are 6 polypeptides mapping in this region, but only ICP10 was consistently isolated with the HSV-2 form of rapid shut-off. There are no known α polypeptides which map in this region.

Virus-specific protein synthesis in HSV-2 infected cells closely parallels that of HSV-1 infected cells. The temporal regulation first described by Honess and Roizman (1974) for HSV-1 infected cells has been shown by Powell and Courtney (1975) and others (Pereira, et al., 1977) to be similar to the regulatory events of HSV-2 protein synthesis. The three major temporal classes of infected cell polypeptides (α, β, and γ) appear with approximately the same cascade regulation kinetics and with apparently the same control mechanisms. The most obvious difference between the protein synthesis kinetics of the two virus types is the rate of protein production:

HSV-2 polypeptides are made at maximal rates sooner and are seen to proceed through the stages of cascade regulation more rapidly than those of HSV-1 (Powell and Courtney, 1975). The polypeptides produced in HSV-2 infected cells appear to have similar structure, modification, and as far as can be ascertained, functional properties to those of the HSV-1 infected cell polypeptides. The migration rates of the HSV-2 polypeptides differ only slightly from their HSV-1 counterparts (Cassai, et al., 1975; Courtney and Powell, 1975; Powell, et al., 1977; Bookout and Levy, 1980).

The immediate-early (α polypeptide) class of HSV-2 polypeptides is defined as the group of polypeptides whose synthesis occurs in the absence of any prior HSV-specific protein synthesis. The α polypeptides of HSV-2 infected cells include ICP4, ICP0, and ICP27, thought to be analogous to the HSV-1 α polypeptides ICP4, ICP0, and ICP27, respectively. A significant difference in HSV-2 and HSV-1 α polypeptide groups is the absence of an identifiable HSV-2 correlate to ICP22 in the HSV-1 system. This polypeptide may either be not coded for in the HSV-2 genome or the HSV-2 protein may migrate at a molecular weight which would make its detection impossible. Although the gene coding for this polypeptide has not been mapped on the HSV genome, there is some evidence for its existence in HSV-2 infected cells. A phosphoprotein which is

isotopically labeled very early in infection with ^{32}P-phosphate appears to be a reasonable candidate for the functional analogue of ICP22 in HSV-2 infected cells (Fenwick, et al., 1980). This phosphoprotein labels only slightly with ^{14}C-amino acids but appears to be heavily phosphorylated in ^{32}P-phosphate-labeled cells. It migrates at a lower apparent molecular weight than would be expected for the HSV-2 analogue of the HSV-1 ICP22.

The functional role of the HSV-2 α polypeptides is yet to be ascertained. This group of polypeptides is thought to be involved in regulating the synthesis of the β class of HSV-2 infected cell polypeptides. This has been clearly demonstrated by Powell and Courtney (1975) and others (Pereira, et al., 1977). These polypeptides have also been associated with the nuclear fraction of infected cells which were separated into crude nuclear and cytoplasmic components. This technique lacks the finesse of a monospecific immunologic tool and can give misleading results due to the elution of nuclear proteins to the cytoplasm or to the adhesion of cytoplasmic proteins to nuclei during the purification process. Studies aimed at defining the HSV-infected cell proteins with DNA-binding capabilities have shown all the α polypeptides to be strongly DNA-binding, thus providing circumstantial evidence to their nuclear location and an idea as to what their functional role may be (Powell and Purifoy, 1976; Purifoy and Powell, 1976).

ICP4. This is the largest of the HSV-2 α class infected cell polypeptides at 182,000 daltons. It is a phosphoprotein which is made very early in infection and is seen to be modified by at least two steps into an apparently higher molecular weight form which can be found in the nuclear fraction of dounce homogenized infected cells. ICP4 is translated in the cytoplasm from an immediate-early mRNA (Easton and Clements, 1980) which is transcribed from the terminal repeats of the S segment of the HSV-2 genome (Figure 2, page 21). The ICP4 gene is diploid, with both copies being transcribed. Intertypic recombinants are available which simultaneously produce the ICP4 molecule from both HSV-1 and HSV-2.

The ICP4 polypeptides from HSV-1 and HSV-2 are thought to be functionally similar (Honess and Roizman, 1974; Powell and Courtney, 1975). Studies using the amino acid analogues canavanine or azetidine produce the same general effects in the HSV-1 and HSV-2 infectious cycles, and the alterations of the ICP4 polypeptides themselves appear to be similar. The step-wise modification has been studied by Wilcox, et al. (1980) with particular emphasis on the phosphorylation of each species with inorganic phosphate. It was found that the phosphorylation was not causally related to the modification itself, but instead, as in the HSV-1 system, appeared to occur independently of it. The ICP4 polypeptide from HSV-2

infected cells was shown to be phosphorylated through both a cyclic and a non-cyclic mechanism. In the HSV-2 system, however, all three forms of ICP4 can be phosphorylated late in infection and also that the ICP4c component is stabily phosphorylated.

A detailed characterization of the type-specificity and ultrastructural location of the HSV-2 ICP4 polypeptide has not been possible due to the lack of monospecific antisera to this polypeptide. Using a monospecific antiserum produced against the HSV-1 ICP4 molecule, Courtney and Benyesh-Melnick (1974) showed the antigen to be type-specific for HSV-1 infected cells. The antiserum reacted with a strong nuclear fluorescence in HSV-1 infected cells but gave no specific fluorescence when used as a stain for HSV-2 infected cells. The reciprocal studies are reported in this thesis.

ICP0 and ICP27. ICP0 and ICP27 (140,000 daltons and 62,000 daltons, respectively) are α polypeptides which are thought to be analogous to the HSV-1 α polypeptides of the same nomenclature. Both are phosphoproteins, but neither appear to have a molecular weight change on pulse labeling or after a chase in the absence of labeled amino acids. They are found in both the nuclei and cytoplasm of HSV-2 infected cells (Pereira, et al., 1977). Wilcox, et al. (1980) examined the DNA-binding properties of ICP27

and found it to be strongly associated with the DNA in DNA-cellulose columns. ICP0 has not been carefully analyzed in this respect. The genes of these two α polypeptides have been mapped by Easton and Clements (1980) and are found in the reiterated sequences of the long segment of the genome (Figure 2, page 21). The gene for ICP0 is diploid.

Accumulation of α Polypeptides and Production of α Polypeptide Antisera

Immediately early polypeptides are made at maximal rates between 4 and 5 hours pi (HSV-1), at which time their rate of synthesis is reduced. These non-structural phosphoproteins are thought to serve a regulatory function associated with the initiation of the subsequent stages of HSV-specific polypeptide synthesis. Because of their purported regulatory function in the infectious cycle, very small amounts of the polypeptides are produced in cells infected with HSV. The low amounts of polypeptide synthesized has made it very difficult to accumulate adequate quantities of the material for quantitative investigations and has indeed made the acquisition of amounts of the antigens sufficient for the immunization of rabbits challenging. Due to the more stringent control of the events in the HSV-2 infectious cycle, the problems of accumulating sufficient quantities of α polypeptide antigens in this system are even more profound.

Because of the inherent problems of working with small quantities of antigenic materials, it has been desirable to find ways to stimulate the overproduction of α polypeptides. Several approaches have been taken. The most straight forward method has involved a simple elaboration of the very procedures used to define the α polypeptides themselves. Cells were infected with HSV in the presence of cycloheximide and incubated for a period of time sufficient to allow the accumulation of α polypeptide mRNA (Ben-Porat, et al., 1974). The cells were then washed free of cycloheximide and actinomycin D was added to allow the selective translation of the α polypeptides as described by Honess and Roizman (1975). A resulting overproduction of α polypeptides was achieved due to the accumulation of specific mRNA as well as to the prolonged translation period itself. This technique appears to be a valuable approach to isolating HSV-1 α polypeptides but is ineffective in the HSV-2 system due either to a lability of the HSV-2 α-message itself or to a dependence of the HSV-2 α polypeptide synthesis on an unstabile cellular factor (Powell and Courtney, 1975). An antiserum directed against the α polypeptides produced in HSV-1 infected cells cultured in this manner (Matis and Rajcani, 1980) yielded a bright nuclear fluorescence in HSV-1 infected cells but not in uninfected cells, indicating the successful accumulation of immunizing quantities of

the α polypeptides. Antisera produced in this manner are interesting but lack the monospecificity for individual polypeptides needed for definitive molecular analyses.

Immediate-early polypeptides have also been shown to accumulate in HSV-infected cells in the absence of virus DNA synthesis (Powell, et al., 1975). Courtney and Benyesh-Melnick (1974) capitalized on this phemonenon to accumulate sufficient quantities of ICP4, shown to be overproduced in a ts mutant (tsB2) by 8- to 10-fold over the amount made in the wild-type infected cell (Courtney, et al., 1976), to immunize rabbits. This antigen was purified to homogeneity by preparative SDS-PAGE. The antisera produced have been used extensively to define the involvement of this α polypeptide with cells lytically (Courtney and Benyesh-Melnick, 1974) or latently (Green, et al., 1981) infected with HSV-1. No ts mutants have been isolated that allow similar accumulation of HSV-2 antigen.

CHAPTER 2

MATERIALS AND METHODS

Tissue Culture and Media

Cells used in this investigation include serially propagated human epidermoid carcinoma No-2 (HEp-2), African green monkey kidney (Vero), rabbit kidney (RK-13), and human embryonic lung (MRC-5) fibroblasts. The cell stocks were maintained in 75 cm^3 plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.) and grown at 37°C in Eagle's medium (Auto-Pow Minimum Essential Media, Flow Laboratories, Rockville, MD) supplemented with 10% newborn calf serum (NCS) or fetal bovine serum (FBS), 100 IU/ml of penicillin, 100 µg/ml streptomycin, 0.075% sodium bicarbonate, and 1X concentrations of vitamins and amino acids (10 X 1 medium). Cells were seeded onto 35-, 60-, or 100-mm plastic plates (Corning) in Eagle medium supplemented with 10% NCS or FBS, 100 IU/ml of penicillin, 100 µg/ml of streptomycin, 0.225% sodium bicarbonate and 1X concentrations of vitamins and amino acids (10 X 3 medium). Cells were grown to confluency in two to three days at 37°C in a humidified incubator with 5% CO_2. After reaching confluency, 10 X 3 medium was removed and replaced with Eagle's medium containing 2% NCS, 100 IU/ml penicillin, 100 µg/ml of streptomycin, 0.225% sodium bicarbonate, and

1X concentrations of vitamins and amino acids (2 X 3 medium). Experiments requiring the use of radioactively labeled amino acids or amino acid analogues were conducted in 2 X 3 medium modified by the omission of the particular amino acid to be substituted.

Chemicals and Radioisotopes

L-α-Amino-γ-[aminooxy]-n-butyric acid (canavanine) and L-Azetidine-2-carboxylic acid (azetidine) were purchased from Sigma Chemical Company, St. Louis, Missouri. L-^{35}S-methionine (specific activity 400-900 Ci/mmole) was purchased from Amersham, Arlington Heights, IL.

Viruses

The 186 strain of HSV-2 (Rawls, et al., 1968) was used throughout this study for both the production of HSV-2 α polypeptide specific immunogens used for rabbit inoculation and for type 2 antigen production for use in all other assays and experiments. The KOS strain of HSV-1 (Smith, 1964) was used to produce all type 1 antigens for use in appropriate assays. Virus stocks were prepared by inoculating monolayers of HEp-2 cells grown in 100 mm petri dishes with a 0.05 multiplicity of infection (moi) for HSV-2 or a 0.1 moi for HSV-1 and adsorbing the virus at 37°C in 5% CO_2 for 1 hour. Following adsorption, the inoculum was removed and 5 ml of 2 X 3 medium were added per plate and the plates were incubated at 34°C

(HSV-2) or 37°C (HSV-1) in humidified air and CO_2. The cells exhibited 4+ cytopathic effect (cpe) in approximately 3 days and were then scraped into the media using sterile rubber policemen. An International Equipment Company (IEC) Model PR-6 centrifuge was then used to pellet the infected cells (2,400 R.P.M. at 4°C for 8 min) and the supernatant was either discarded (HSV-2) or a portion of it retained for the resuspension of the infected cell pellet (HSV-1). The pelleted cells were then suspended in sterile H_2O (HSV-2) or infected cell supernatant (HSV-1) at a concentration of 10^7 cells per ml and disrupted by ultrasonic vibration at 10 kHz for 30 sec using a Sonicator (Heat Systems - Ultrasonics, Inc.) brand sonic oscillator. The virus stock was clarified by centrifugation at 1000 R.P.M. for 5 min in an IEC PR-6 centrifuge and stored in 2-5 ml aliquots at -70°C. Sterility of the virus stocks was determined by inoculating a few drops of the final stock preparation onto sheep red blood cell agar plates and incubating at 37°C overnight.

All virus stocks were filtered by the plaque assay technique using confluent monolayers of Vero cells grown at 37°C in 60 mm plastic plates. Assays were done using serial ten-fold dilutions of stocks which had been frozen and thawed once. Several 0.1 ml aliquots of appropriate virus dilutions were inoculated onto duplicate plates and adsorbed at 37°C with intermittent rotation to facilitate

even dispersion of the inoculum. After the adsorption period, the inoculum was removed, 10 X 3 medium with 2% methylcellulose (Dreesman and Benyesh-Melnick, 1967) was overlayed and the plates were returned to the 37°C incubator for 2 days. Buffered neutral red solution was then added to the medium to be used as a vital dye to stain the living cells in the monolayer. The plates were incubated overnight and the titers of the virus stock were determined by counting plaques the next day.

Infection, Labeling, and Harvesting of Infected Cells

Cell monolayers approaching confluency were washed with phosphate buffered saline (PBS, see Appendix), pH 7.4, and infected at an moi of 20-40 pfu/cell. After adsorption for 1 hour with occasional rotation in a 37°C humidified CO_2 incubator, the inoculum was removed and media appropriate for the particular experiment added. Experiments were carried out at 37°C in a humidified air atmosphere with 5% CO_2, unless otherwise noted.

Media used for labeling experiments and production of α polypeptide antigens consisted primarily of 2 X 3 medium appropriately modified for the experiment. This modification usually consisted of an omission of various combinations of amino acids and the substitution of either isotopically-labeled amino acids or amino acid analogues. Polypeptides synthesized in the presence of canavanine were labeled with ^{35}S-methionine (25-50 μCi/ml) in 2 X 3 medium

lacking arginine (of which canavanine is an analogue), and methionine. Cells are infected in the presence of 2.8 mM canavanine and isotopically labeled at various times post infection. Polypeptides synthesized in the presence of azetidine were labeled with ^{35}S-methionine (25-50 µCi/ml) in 2 X 3 medium lacking proline, hydroxyproline (of which azetidine is an analogue), and methionine. Azetidine (4 mM) was added at the time of infection and cells were labeled at various times post infection in the presence of the inhibitor.

HSV-infected cells were harvested after an appropriate incubation period by scraping the cells into the media with a rubber policeman and pelleting the cells in a table-top medel IEC clinical centrifuge at 3350 R.P.M. for 5 min. The pelleted cells were washed 3X in cold (4^{o}C) PBS, pH 7.4, and suspended in 0.2 ml (60 mm plate) or 0.4 ml (100 mm plate) of glass distilled water. The cells were then disrupted by ultrasonic vibration at 10 kHz for 2 min and stored frozen at -20^{o}C in polystyrene snap-cap tubes.

Dounce Homogenization of HSV-2 Infected Cells

HEp-2 cells were infected with HSV-2 and labeled with ^{35}S-methionine (25-50 µCi/ml) from 2-6 hrs pi. Cells were scraped into the medium and washed 3X in cold (4^{o}C) PBS, pH 7.4. Infected cells were pelleted in an IEC clinical centrifuge and suspended in Reticulocyte Standard

Buffer (RSB; 0.1 M Tris, 0.01 M KCl, 0.0015 M $MgCl_2$) (Courtney, et al., 1971) at a concentration of approximately 1 X 10^7 cells per ml. After a 10 min incubation at 0°C, cells were homogenized with 20 strokes of an ice cold Dounce homogenizer and centrifuged at approximately 1600 R.P.M. for 4 min in an IEC clinical centrifuge. The supernatant was frozen at -20°C to be used as the cytoplasmic fraction. The nuclei were washed in RSB and suspended and vortexed for 5 sec in RSB made 0.5% (w/v) deoxycholate and 1.0% (v/v) Tween 40. The nuclei were then pelleted, washed twice in RSB, suspended in sterile glass distilled water and disrupted by ultrasonic vibration for 2 min at 10 kHz. The nuclear fraction was stored at -20°C.

Antigen Preparation

HEp-2 cells were infected with HSV-2 strain 186 in the presence of 2.8 mM L-canavanine and cultured for 6-8 hrs at 37°C in humidified air and 5% CO_2. ^{35}S-methionine was used to isotopically label approximately one-fourth of the infected cell cultures. Cells were harvested as previously described and were suspended in 0.1 ml H_2O per 100 mm plate. The isotopically-labeled infected cell preparations were mixed with the unlabeled cells and stored at -20°C.

Preparative SDS-Polyacrylamide Gel Electrophoresis

Preparative, cylindrical and analytical polyacrylamide gel electrophoresis was performed using the discontinuous buffer system of Davis (1964) and Ornstein (1964) with the modification by Dimmock and Watson (1969) for the inclusion of sodium dodecyl sulfate (SDS). Preparative electrophoresis was performed using a Buchler preparative gel electrophoresis unit (polyprep) (Buchler Instruments, Inc., Fort Lee, N.J.) containing 40 to 60 ml of separating gel and a stacking gel volume equal to or greater than 1.5 times the amount of sample added. The separating gel was prepared in 0.38 M Tris-HCl (pH 8.8-9.0), and consisted of the acrylamide monomer (6.0-10.0% w/v), N,N,N',N',tetramethylethylenediamine (TEMED; 0.3% v/v), ammonium persulfate (0.07%, w/v), SDS (0.1%, w/v) and N,N'-methylene-bis-acrylamide (BIS; 0.176%, w/v). The acrylamide monomer concentration was varied to optimize the resolution of polypetides of varying molecular weights. The stacking gel was prepared in 0.06 M Tris-HCl (pH 6.6-6.8) and consisted of the acrylamide monomer (2.56%, w/v) BIS (0.64%, w/v), TEMED (0.06%, v/v), riboflavin (0.0005%, w/v) and SDS (0.1%, w/v).

Samples to be used for polypeptide purification were stored at -20^{o}C until the time for electrophoresis. The cell lysates were solubilized immediately before electrophoresis by boiling for 2 min in 0.05 M Tris-HCl

(pH 6.7), 1% SDS, 1% β-mercaptoethanol, 0.5 M urea and 3% sucrose with bromphenol blue added as a tracking dye. After solubilization, the sample was layered onto the stacking gel and electrophoresed at a constant current of 60 mAmp The upper, lower, and elution buffer reservoirs contained Tris-glycine buffer composed of 0.025 M Tris, 0.19 M glycine and 0.1% SDS at a final pH of 8.6.

Electrophoretically separated proteins were eluted from the bottom of the gel and were collected in 4 ml fractions of elution buffer every 6 min for 12 to 14 hrs. Isotopically-labeled polypeptide bands were located by aliquoting 100 µL of alternate fractions into 3 ml of zylene based Aqueous Counting Scintillant (ACS) (Amersham Corp., Arlington Heights, IL.) and counting the samples in a Beckman LS-7000 liquid scintillation counter. Peak fractions were collected, pooled and concentrated to approximately a 1.5 ml volume by ultrafiltration using an Amicon ultrafiltration cell with a PM-10 membrane (Amicon Corporation, Lexington, MA.). The purity of each concentrated polypeptide was assayed by examining a small aliquot of the sample in an analytical polyacrylamide gel electrophoresis system. The concentrated polypeptides were stored at -70^{o}C until an appropriate time for banding onto cylindrical polyacrylamide gels.

Cylindrical SDS-Polyacrylamide Gel Electrophoresis

Cylindrical gel electrophoresis was performed using the same gel reagents and concentrations described for preparative gel electrophoresis with all cylindrical separating gels made at a 7% acrylamide monomer concentration. Cylindrical gels were used for banding the purified antigens to be used for immunization and also for estimating the amount of protein associated with each band. The gels were polymerized in 0.6 X 12 cm glass tubes containing 2.0-2.5 ml separating gel and approximately 0.5 ml stacking gel. Upper and lower Tris-glycine buffers used were the same as those used in preparative electrophoresis. Approximately 330 µL of sample were layered onto the stacking gel and electrophoresed at 4 mAmp per gel until the dye band reached the bottom of the gel.

Following electrophoresis, all but one of the multiple gels of each sample were frozen at -20°C. The remaining gel was stained overnight at 37°C with 0.25% Coomassie brilliant blue made in destain solution (25% methanol and 7% acetic acid). The gels were destained at 37°C using several changes of destain solution until the background was transparent. The relative migration rates of the leading and trailing edges of the polypeptide bands were then measured using the stacking gel/separating gel interface and dye band as reference points. A 1.0 cm section containing the polypeptide band was then excised

from each of the frozen, unstained gels and stored at -20°C until used for the immunization of rabbits.

The remaining stained gel was used to densitometrically estimate the amount of protein contained in each band. Standards used for determining protein concentrations were prepared by making serial two-fold dilutions of bovine serum albumin (BSA) with final concentrations ranging from 500 µg/ml to 15.62 µg/ml. Each BSA dilution was prepared for cylindrical gel electrophoresis and stained in the same manner described for HSV-2 purified proteins. The gels were scanned using an E-C Apparatus (St. Petersburg, FL.) densitometer with a 540 nm visible light range dielectric filter and an Omniscribe Recorder (Houston Instruments, Austin, TX). Relative band densities were obtained by estimating the area beneath the curves (directly related to the weight of paper circumscribed by each peak, as determined by weighing peaks on a Metler H-30 analytical scale). The known standard concentrations were then plotted against their corresponding peak weights and the concentration of the purified HSV-2 polypeptide bands were obtained from their peak weights by using linear regression.

Analytical SDS-Polyacrylamide Gel Electrophoresis

Analytical polyacrylamide gel electrophoresis (PAGE) was performed on vertical 0.75 X 100 X 160 mm slab gels with 5 mm (20 wells) or 10 mm (10 wells) sample wells.

The separating gels were prepared as described for the preparative SDS-PAGE system, with all gels having a 7% acrylamide monomer concentration. The stacking gel was prepared in 0.06 M Tris-HCl buffer (pH 6.6-6.8) and consisted of the acrylamide monomer (3.86%, w/v), BIS (0.96%, w/v) TEMED (0.06%, v.v), riboflavin (0.0005%, w/v) and SDS (0.1%, w/v). All gels were polymerized and used on the same day.

Samples were solubilized immediately prior to electrophoresis as previously described for the preparative SDS-PAGE system. Gels had 10-30 µL (10 mm sample wells) or 5-10 µL (5 mm sample wells) of sample loaded per well and were electrophoresed at a constant current of 15-20 mAmp per gel in a Hoefer electrophoretic unit (Hoefer Scientific, San Francisco, CA.). Tris-glycine upper and lower electrophoresis buffers were the same as described for the preparative SDS-PAGE system.

Following electrophoresis, the gels were fixed in destaining solution for 5-10 min and dried under vacuum on 3M Whatman chromatographic paper in a Hoefer Scientific Slab Gel Dryer. For autoradiography, dried gels were exposed to either Kodak NS-2T no screen X-ray film or to pre-flashed Kodak XR-5 X-Omat X-ray film.

Radioimmunoprecipitation

HSV-2 infected cell antigens were prepared as previously described and then solubilized for

radioimmunoprecipitation (RIP) by making the sample 1% in sodium deoxycholate, 1% in Tween 40, and 0.1% in SDS. The sample was then incubated for 1 hr at 0°C. Solubilized samples were then ultracentrifuged for 1 hr at 25,000 R.P.M. in a Beckman Type 30 fixed angle rotor and the supernatant was used as the antigen for RIP.

Immunoprecipitation from detergent-solubilized extracts was performed in glass tubes in a total volume of 150 µL comprised of 100 µL of 0.5% TX-100 (v/v) in TNE (0.15 M NaCl, 0.0005 M EDTA, and 0.05 M Tris, pH 7.4) mixed with 25 µL of solubilized extract and 25 µL of antiserum. The reaction mixture was incubated at 37°C for 1 hr in a constant-shaking water bath. After an additional 1 hr incubation at 4°C, 20 µL of Pansorbin (Staph A, Calbiochem-Behring Corp., La Jolla, CA.) or 100 µL of a 10% solution of Staph A Sepharose diluted in TNE was added to the mixture and incubated overnight at 4°C. The immune complexes were pelleted from solution in an IEC Clinical Centrifuge at 2,400 R.P.M. and washed 5 times in cold TNE (4°C). The final pellet was solubilized in 35 µL of 2% SDS, 1M Urea, 2% β-mercaptoethanol, 3% sucrose, 0.06 M Tris-HCl and 0.06% TEMED (v/v) (RIP-SUM) at 37°C for 1 hr. The sample was then boiled for 30 sec and the released Staph A was pelleted in a clinical centrifuge. Five µL of the resulting supernatant containing the immunoprecipitated protein was assayed for radioactivity and the

remainder of the sample was loaded directly onto an analytical slab gel and electrophoresed as described earlier.

Production of Antisera in Rabbits

All antigens used to elicit monospecific antisera to purified α polypeptides were electrophoresed into multiple cylindrical polyacrylamide gels as described above. For the primary immunization of each rabbit, the excised bands of two gels were macerated and emulsified in a 1 ml total volume of equal parts sterile PBS and Freund's complete adjuvant (GIBCO, Grand Island, N.Y.). Rabbits were injected with approximately 0.5 ml of the antigen preparation into each of the hind footpads. Antigen preparations for booster injections contained one macerated gel segment emulsified in 0.5 ml of an equal mixture of sterile PBS and Freund's incomplete adjuvant. Booster injections were given intramuscularly with half the preparation going to the gastrocnemius muscle of each calf. Booster injections were made at two week intervals.

Positive control serum used in these studies was prepared by immunizing rabbits with cell extracts of HSV-2 infected RK-13 cells exhibiting 4+ cpe. This antigen was prepared using cells infected as described above and cultured at 34^{o}C in 2 X 3 maintenance medium with 2% rabbit serum substituted for NCS. Infectious virus in the primary immunizing preparation was inactivated by treatment with 1% formalin (v/v, final concentration) for

1 hr at 37°C followed by an overnight incubation at 4°C. The primary immunization was performed as described above and booster injections were performed using antigen preparations containing infectious virus. Normal rabbit serum (NRS) used in these studies as a negative control serum was prepared by repeatedly injecting a rabbit with 1 cm segments of a polyacrylamide gel containing no protein.

Animals used in this study were adult, male, New Zealand white rabbits. All rabbits were pre-bled prior to immunization and were bled weekly starting approximately eight weeks after the primary immunization. All bleedings were done from the peripheral ear vein under vacuum using a rabbit ear bottle (Bellco Glass Co., Inc., Vineland, N.J.). Blood was allowed to clot for 1 hr at 37°C and the clot allowed to retract overnight at 4°C. The serum was then extracted and centrifuged for 10 min at 3500 R.P.M. in an IEC model PR-6 centrifuge to remove all contaminating cells and stored in aliquots at -20°C.

Indirect Immunofluorescence

The indirect immunofluorescence test of Porter, et al. (1969), with slight modification, was used in these studies. Prior to use each antiserum was first adsorbed with 0.1 gm of rabbit liver powder per ml by shaking for 1 hr at a 37°C water bath and then incubating overnight

at 4°C. The rabbit liver powder was removed by centrifugation at 100,000 X g for 15 min and then further adsorbed with a mixture of HEp-2 and Vero cells. The 1 X 10^8 cells of each type were ultrasonically disrupted and added to the antiserum and the procedure was repeated as described above for the rabbit liver powder. After clarification of the antiserum by ultracentrifugation, 1:2 or 1:4 dilutions in PBS were made immediately prior to use. The anti-HSV-1, anti-HSV-2, and anti-polyacrylamide (negative control) antisera were diluted 1:10 in PBS and the fluorescein isothiocyanate-conjugated goat anti-rabbit serum (FITC-GAR) (Hyland Laboratories, Costa Mesa, CA.) was diluted 1:20 in PBS immediately prior to use.

Cells for use in immunofluorescence studies were seeded onto coverslips (approximately 5 X 10^5 cells per coverslip) and grown at 37°C in a humidified air and CO_2 atmosphere. The cells were infected at an moi of 0.5 to 1.0 pfu/cell either in the presence or absence of amino acid analogues and harvested at appropriate times post infection. The coverslips were then washed 3 times in PBS, pH 7.4, and air dried. The cells were then fixed to the coverslips by a 10 min wash in cold acetone and air dried again. At this point, coverslips were either stained immediately or stored in a dessicator at 4°C. Coverslips were rehydrated in PBS for 5 min immediately prior to staining with the primary antibody. Rehydrated

coverslips were placed in a humidified chamber and stained at 25°C for 30 min with hyperimmune antisera specific for HSV-2 polypeptides. The second antibody, FITC-GAR, was applied after the cells had been rinsed 3 times in PBS and was incubated for an additional 30 min in the humidified chamber. Following staining, the coverlsips were rinsed in PBS, dipped in distilled water to dilute buffer salts, air dried and mounted on microscope slides in 50% glycerol. Stained coverslips were examined and photographed at a 400X magnification using a Nikon Fluophot microscope (Nippon Kogaku, Tokyo, Japan) with a Nikon Plan Apo 40 lens and Kodak Ektachrome daylight ASA 400 film.

CHAPTER 3

EXPERIMENTAL RESULTS

Immediate-early polypeptides from HSV-2 infected cells were resolved and purified by preparative SDS-PAGE. Monospecific antisera to each protein were produced in rabbits and these antisera were used to detect the intracellular location and the immunological specificity of the α polypeptides produced in HSV-infected cells. The results of this study will be presented in three sections: 1) preliminary investigations on the HSV-2 infected cell α polypeptides, 2) the production and characterization of rabbit α polypeptide antisera, and 3) the use of the monospecific α polypeptide antisera to study the properties of α polypeptide expression in HSV-2 infected cells.

Preliminary Investigations

HSV-infected cell polypeptides. HSV-2 infected cultures isotopically labeled at various times post infection have been utilized to demonstrate the appearance of the α polypeptide class of HSV-2 infected cell polypeptides (Figure 3). HEp-2 cells inoculated with a moi of 190 pfu/cell of HSV-2 were used for this experiment. The infected cells were grown at 37^{o}C in 2 X 3 medium lacking methionine and pulse labeled with ^{35}S-methionine

Figure 3. Analysis of HSV-2 polypeptides made at various stages during the virus replication cycle. HEp-2 cells were infected with HSV-2 and radioactively labeled with ^{35}S-methionine (25 μCi/ml) for 1 hr intervals as indicated by the time period (hr post infection) above each gel lane. At the end of the 1 hr pulse the cultures were harvested and prepared for analysis by slab gel electrophoresis. The positions of three alpha polypeptides, ICP4, ICP0, and ICP27, are indicated.

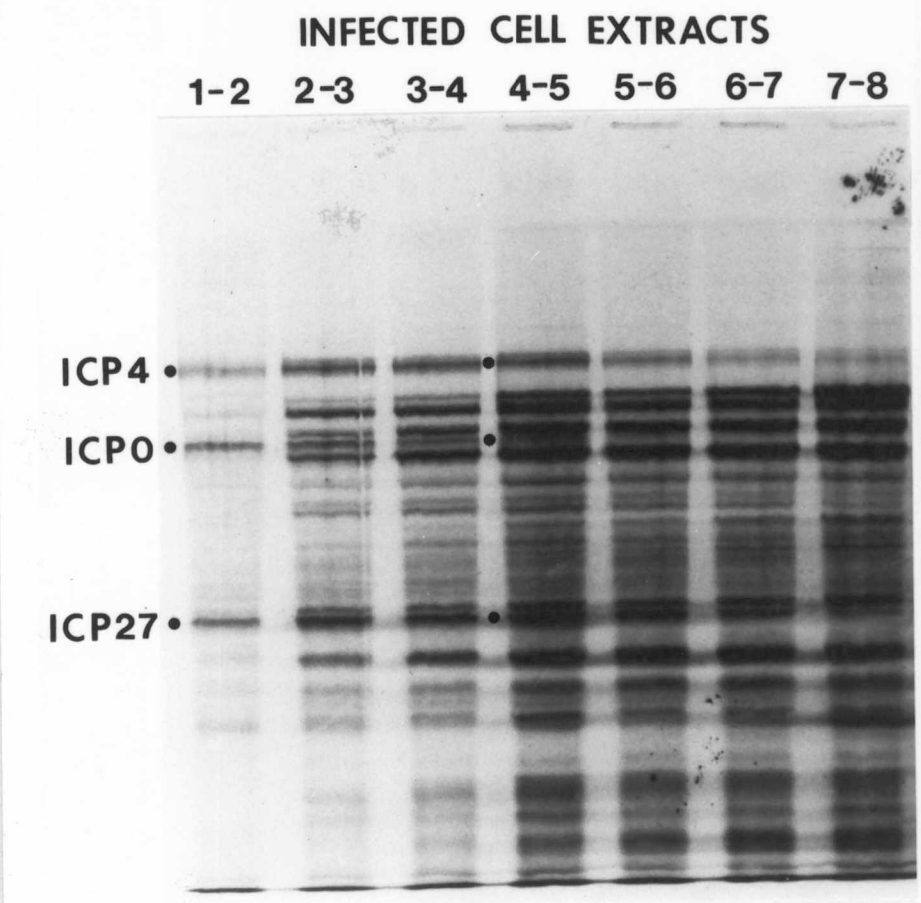

Figure 3.

for 1 hr at various times post infection. Cultures were harvested immediately after the 1 hr pulse and stored at -20°C until analyzed by SDS-PAGE.

The kinetics of synthesis of the HSV-2 α polypeptides can be seen in Figure 3. The HSV-2 α polypeptides (ICP4, ICP0, and ICP27) were seen to be the predominant species made during the first two hours of infection (see pulse from 1 to 2 hrs, Figure 2), at which time the inhibition of host cell polypeptides can also be seen. The effects of the HSV polypeptide control mechanism is shown by observing the decline (note sequential pulse periods) in the synthesis of each of the α polypeptides. A comparison of the intensities of the bands produced at each pulse period indicate that the amounts of ICP4 and ICP27 synthesized at each period appear to be similar. ICP4 and ICP27 were produced maximally between 2 and 5 hrs pi and were severely inhibited during the 7 to 8 hrs pi pulse period. The amounts of ICP0 produced during a 1 hr pulse period appeared to be maximal at earlier times in the infection (between 1 and 3 hrs pi) and its synthesis was no longer detectable by 6 hrs pi. ICP6 (Figure 1, page 17), although considered by some investigators to be a member of the HSV-2 α polypeptide class, was synthesized at maximal amounts throughout the labeling period and appears to follow the kinetics of β-polypeptide synthesis. The

synthesis of this polypeptide appeared to reach its maximum rate between 4 and 5 hrs pi and continue being produced at this rate through 8 hrs pi.

Accumulation of HSV-2 α polypeptides. Rabbit antiserum monospecific for VP175, an HSV-1 α polypeptide, has been made previously in our laboratory. The problems associated with this task due to the exceedingly small quantities of the antigen produced in infected cells were circumvented by the use of a ts mutant which overproduced the polypeptide. A ts mutant of HSV-1 KOS, tsB2, when grown at the nonpermissive temperature (39°C) produces an abnormally large amount of VP175 (Figure 4). This overproduction of VP175 provided the opportunity to purify sufficient quantities of the VP175 antigen to immunize rabbits. The data collected using this antiserum has been reported elsewhere (Courtney and Benyesh-Melnick, 1974; Cabral, 1980). However, no ts mutants of HSV-2 have been isolated which overproduce the α polypeptides, therefore, a much more laborious approach had to be used.

The problem of obtaining sufficient quantities of the HSV-2 α polypeptides for immunization was approached by two separate techniques: one used polypeptides produced in infected cells grown in the absence of inhibitors and another used polypeptides produced in infected cells cultured in the presence of the arginine analogue,

Figure 4. Autoradiogram of infected cell polypeptides synthesized in cells infected with HSV-1 tsB2. HEp-2 cells were infected with tsB2 and labeled with ^{35}S-methionine (25 μCi/ml) from 2 to 6 hrs pi. Cultures were incubated at either the permissive (34°C) or non-permissive (39°C) temperature. At 6 hrs pi cultures were harvested and prepared for analysis by slab gel electrophoresis.

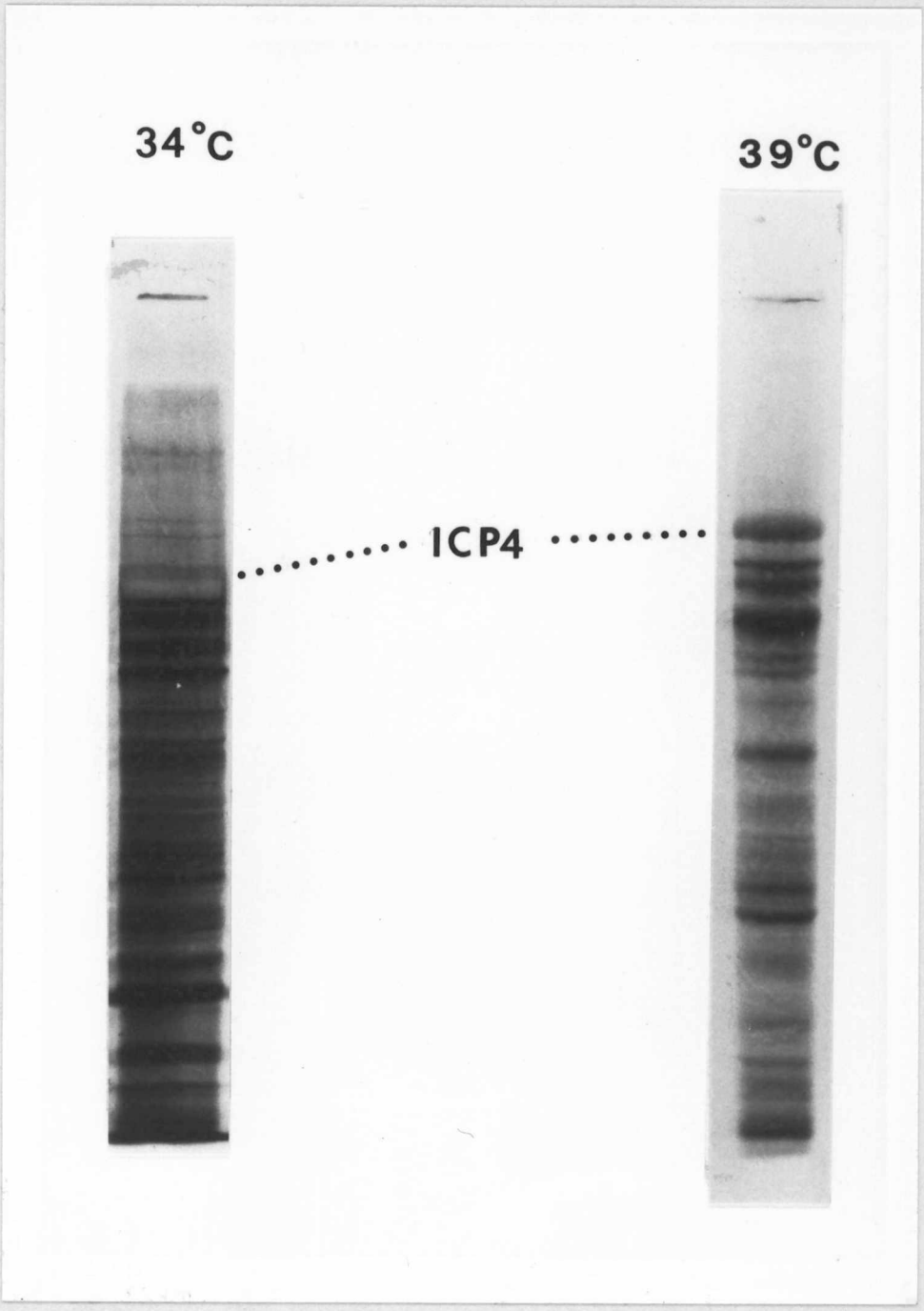

Figure 4.

canavanine. The two approaches were carried out concurrently at all stages from antigen production to antisera collection.

The approach using HSV-2 α polypeptides produced in the absence of the inhibitor was based upon fractionating cells into nuclear and cytoplasmic components in an effort to enrich for the nuclear-associated α polypeptides. HEp-2 cells infected with HSV-2 at an moi of 30 pfu/cell were homogenized with 20 strokes of an ice cold dounce homogenizer, processed as described in Materials and Methods and the resulting nuclear and cytoplasmic fractions were analyzed by SDS-PAGE (Figure 5). ICP4 and ICP0 can be seen associated predominantly with the nuclear fraction, whereas ICP27 is found in both the nuclear and cytoplasmic fractions. The nuclear fractions thus collected from infected cells were applied to a preparative SDS-PAGE system (see Materials and Methods) and a representative profile of the eluted polypeptides is shown in Figure 6. These polypeptides were purified to single band homogeneity and used to immunize rabbits.

The approach utilizing amino acid analogues had the dual advantage of allowing the prolonged production (and thus, an accumulation) of the α polypeptides and also of inhibiting the production of other HSV-2 polypeptides which could interfere with the purification of the α polypeptides. The effects of canavanine and

Figure 5. A comparison of HSV-2 polypeptides associated with nuclear and cytoplasmic cell fractions. HEp-2 cells were infected with HSV-2 and radioactively labeled with ^{35}S-methionine (25 μCi/ml) from 2 to 6 hrs pi. At 6 hrs pi the cells were harvested and separated into cytoplasmic and nuclear fractions by dounce homogenization as described in the Materials and Methods. Aliquots of the cytoplasmic and nuclear fractions were then prepared for analysis by SDS slab gel electrophoresis as described in Materials and Methods.

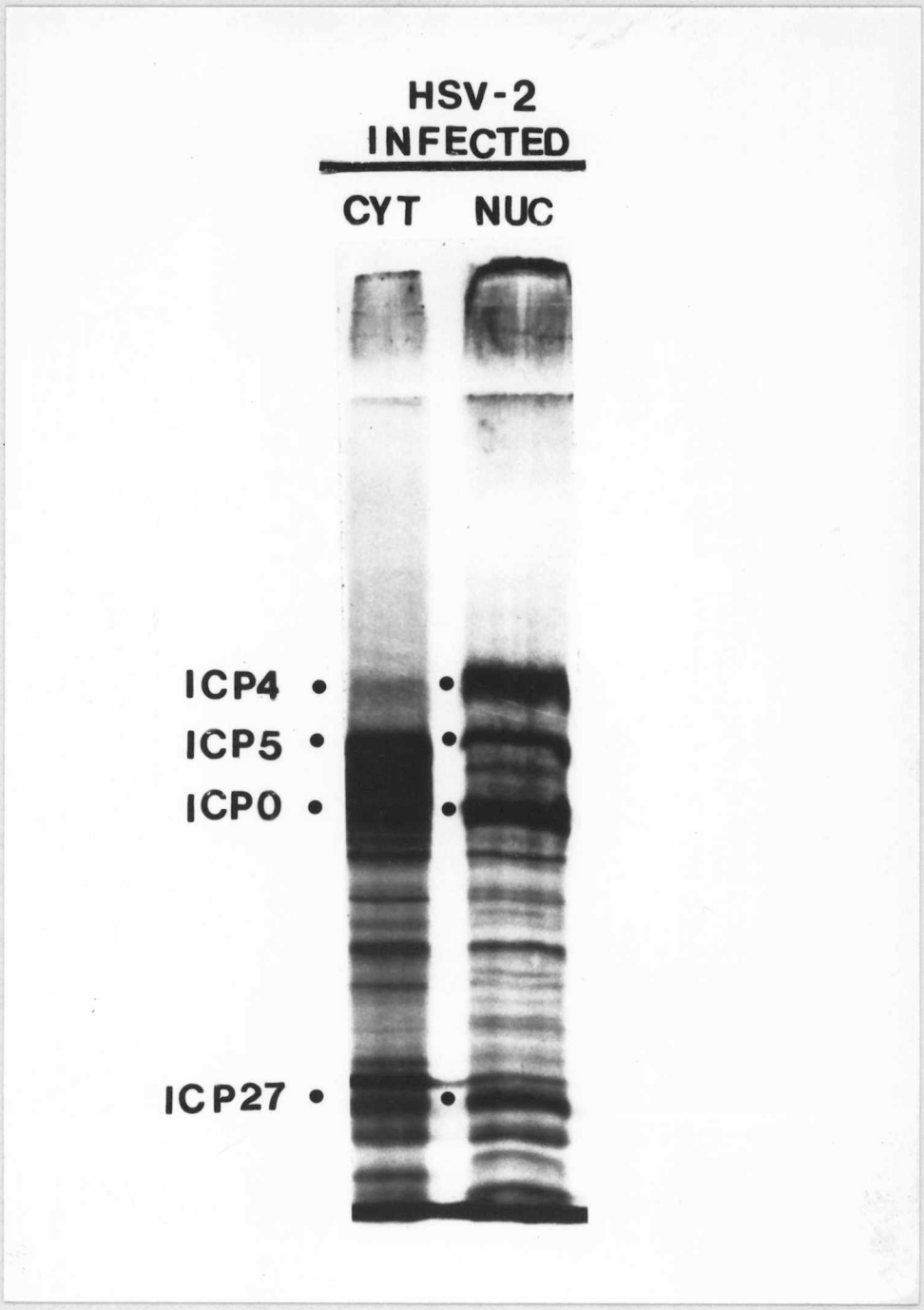

Figure 5.

Figure 6. Preparative SDS-PAGE elution profile from the nuclear fraction of HSV-2 infected cells. HEp-2 cells were infected with HSV-2 and labeled with ^{35}S-methionine (25 µCi/ml) from 2 to 6 hrs pi. At 6 hrs pi the infected cells were harvested and Dounce homogenized to separate the cytoplasmic and nuclear fractions as described in the Materials and Methods. The nuclear fraction was prepared for SDS-PAGE and electrophoresed on a 6% acrylamide separating gel. The bars indicate the pooled fractions which contained polypeptides ICP4 and ICP0.

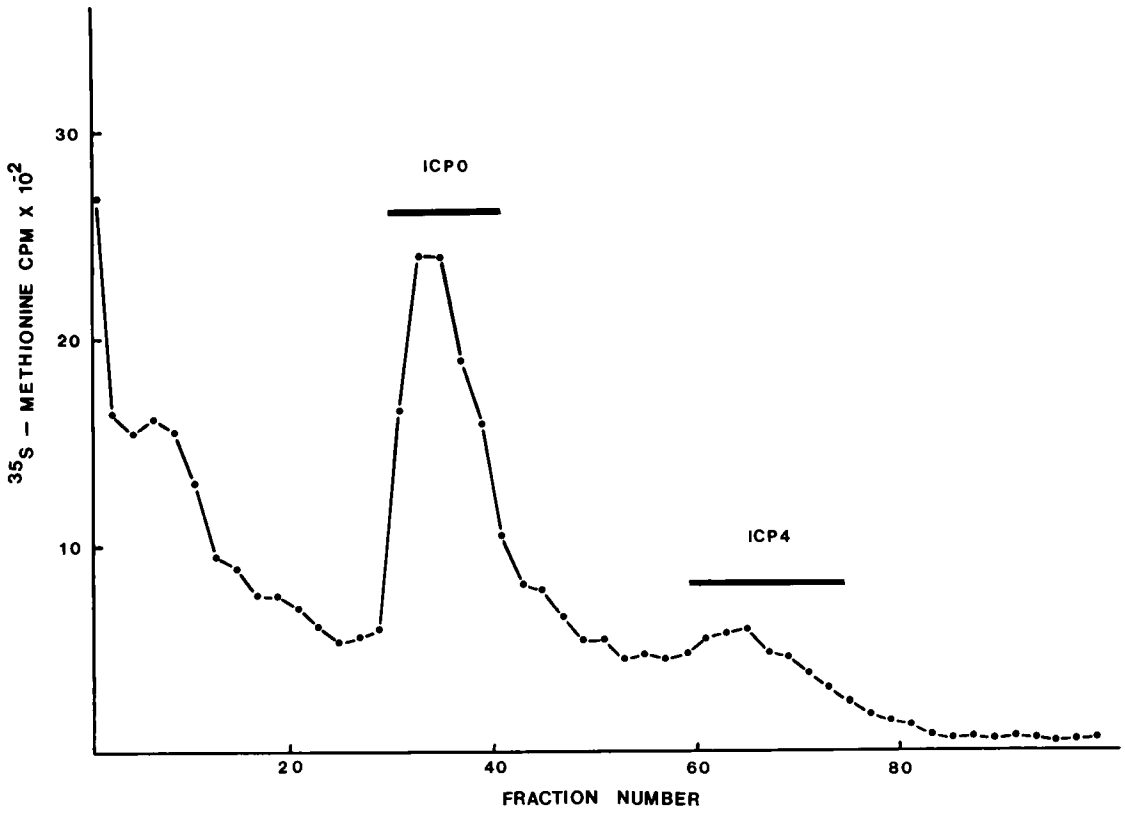
30
20
10
^{35}S – METHIONINE CPM X 10^{-2}
ICP0
ICP4
20
40
60
80
FRACTION NUMBER

Figure 6.

azetidine on the kinetics of HSV-2 polypeptide synthesis is shown in Figures 7 and 8. Although the effects of the two amino acid analogues on HSV-2 polypeptide synthesis were similar, there were certain characteristic differences that should be mentioned. Polypeptides produced in cells cultured in the presence of canavanine migrated as diffuse bands with a higher apparent molecular weight than those synthesized in the absence of the inhibitor (Figure 7). In addition, in the presence of canavanine certain β polypeptides (ICP6) as well as one γ polypeptide (ICP5, the major capside protein) were synthesized in significant amounts. It can also be seen (Figure 7) that in the presence of canavanine, the duration of synthesis of α polypeptides is prolonged compared to the cultures in which no canavanine was present. The most dramatic differences can be seen by comparison of the rates of synthesis of ICP4 and ICP27 grown in cultures with and without canavanine and labeled from 7 to 8 hrs pi. Polypeptides synthesized in the presence of azetidine were observed to migrate on polyacrylamide gels as very discrete bands of apparently the same molecular weight as the polypeptides synthesized in the absence of the inhibitor. Of particular interest was the apparent inhibition of the processing of ICP4 from the lower molecular weight component to the forms having a higher apparent molecular weight (Figure 8). This is a very stringent inhibition and at no time when labeling

Figure 7. Autoradiogram of HSV-2 polypeptide synthesis in the presence and absence of canavanine. HEp-2 cells were infected with HSV-2 and cultured in the presence (+) or absence (-) of 2.8 mM canavanine. At various times after infection (as indicated by the time intervals listed above the gel lanes) the cells were isotopically-labeled with ^{35}S-methionine (25 μCi/ml). At the end of the 1 hr pulse the cultures were harvested and prepared for analysis by slab gel electrophoresis. The mock-infected control cultures (CON) were isotopically labeled for 2 to 8 hrs pi and harvested at 8 hrs pi.

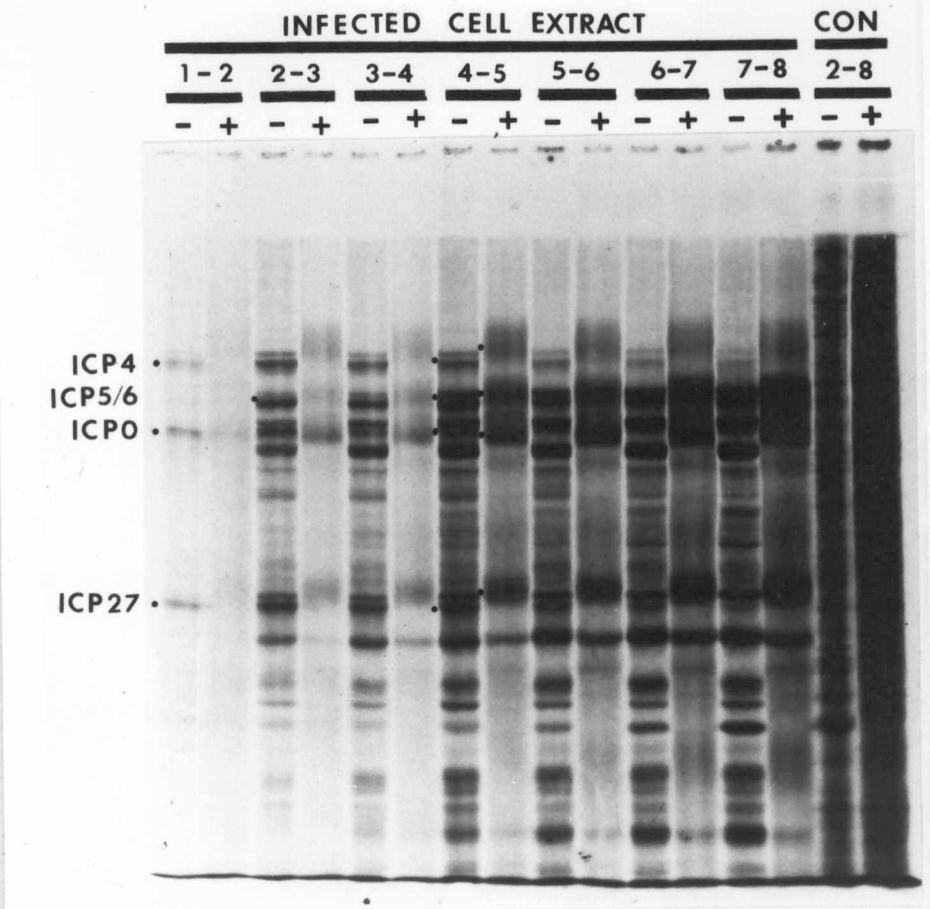
INFECTED CELL EXTRACT
CON
1-2
2-3
3-4
4-5
5-6
6-7
7-8
2-8
- + - + - + - + - + - + - + - +
ICP4
ICP5/6
ICP0
ICP27

Figure 7.

Figure 8. Autoradiogram of HSV-2 polypeptide synthesis in the presence and absence of azetidine. HEp-2 cells were infected with HSV-2 and cultured in the presence (+) or absence (-) of 4 mM azetidine. At various times after infection (as indicated by the time intervals listed above the gel lanes) the cells were isotopically-labeled with ^{35}S-methionine (25 µCi/ml). At the end of the 1 hr pulse the cultures were harvested and prepared for analysis by slab gel electrophoresis. The mock-infected control cultures (CON) were isotopically labeled for 2 to 8 hrs pi and harvested at 8 hrs pi.

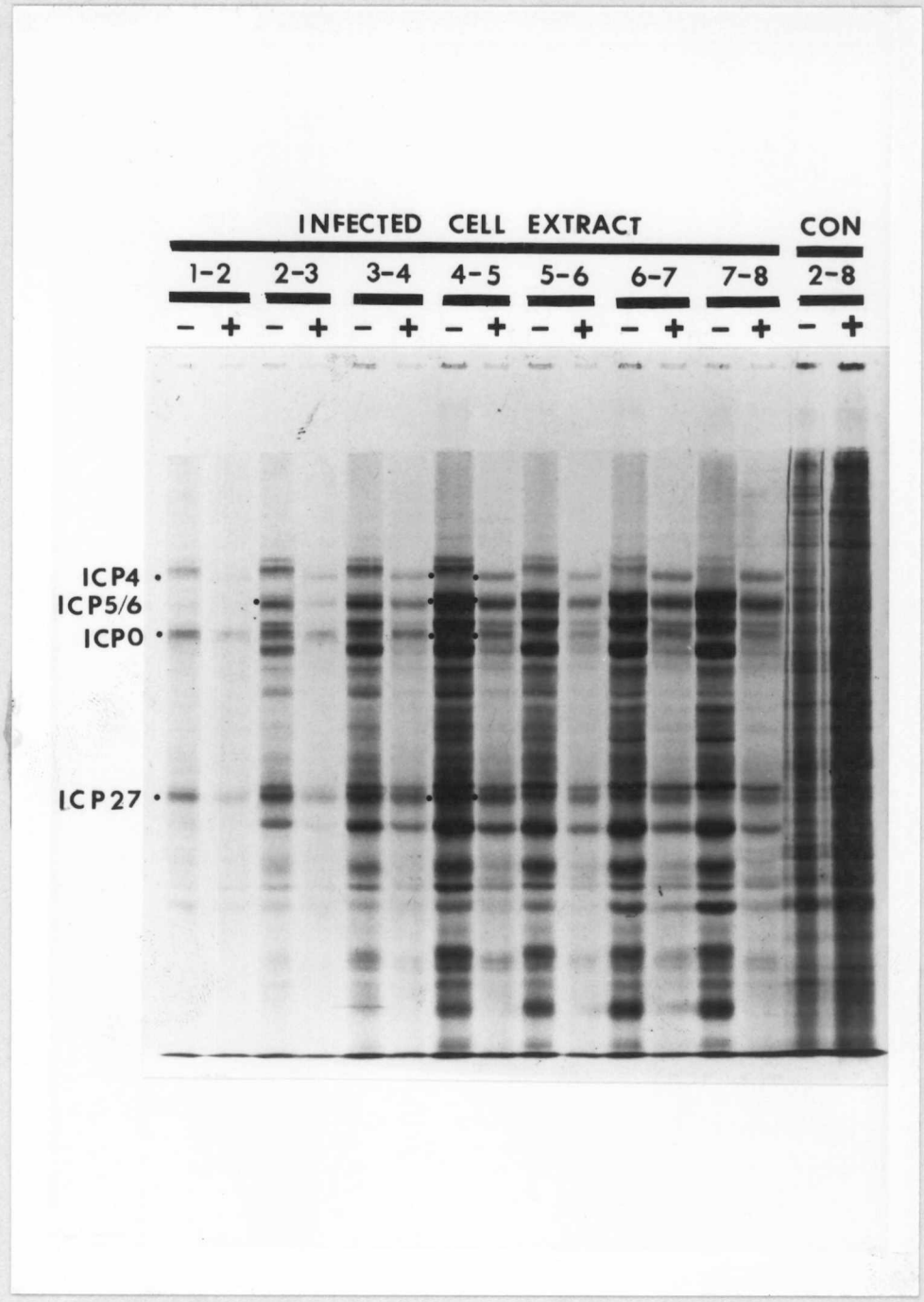
INFECTED CELL EXTRACT
CON
1-2
2-3
3-4
4-5
5-6
6-7
7-8
2-8
- + - + - + - + - + - + - + - +
ICP4
ICP5/6
ICP0
ICP27

Figure 8.

in the presence of azetidine was this inhibition seen to be overcome. In the presence of azetidine some β polypeptides were synthesized; however, the production of the major capsid protein (ICP5) was not detected. The continued production of α polypeptides through the 7 to 8 hrs pi pulse period in the presence of azetidine was also demonstrated in Figure 8. ICP4, ICP0, and ICP27 were still being made in significant amounts at this time, whereas the ICP4 and ICP0 polypeptides were not detected in the cells cultured in the absence of the inhibitor and labeled at the same time post infection.

The use of amino acid analogues has thus allowed the prolonged synthesis and accumulation of the HSV-2 α polypeptides. The α polypeptides produced in the presence of canavanine and azetidine are nonfunctional due to their abnormal tertiary conformations, with the conformational changes induced by azetidine being greater than those induced by canavanine. The greater conformational changes induced by azetidine results in the more stringent inhibition expressed as the inhibition of the processing steps of ICP4 and the inhibition of the synthesis of the ICP5 major capsid protein.

Monospecific Antisera Production and Characterization

SDS-PAGE separation of HSV-2 infected cell α polypeptides.

Preparative SDS-PAGE has been used in our

laboratory for the purification of several HSV-specific polypeptides. This technique has the same very high resolving power which makes analytical SDS-PAGE an invaluable molecular tool. Polypeptides in the molecular weight range of 50,000 to 150,000 are best resolved when eluted from the column in fractions 40 through 80 after the elution of the bromphenol blue marker. Because of this feature, it was necessary to determine the optimum acrylamide gel concentration and gel volume which would result in the best resolution of the proteins. Proteins eluted in approximately the first 25 fractions following the elution of the bromphenol blue dye marker were found to be a mixture of several polypeptides with a broad molecular weight range. This was demonstrated best by comparing the ICP27 peaks isolated from preparative gels with different percent acrylamide monomer compositions (Figure 9). When eluted from a 7% acrylamide gel (Figure 9C) the ICP27 component is easily seen to be separated from smaller molecular weight polypeptides, but when the same "purified" fraction is electrophoresed on a slab gel or on a higher percentage preparative gel (Figure 9D) it is seen to be contaminated with cellular polypeptides, the most prominant of which is probably actin. Other problems taken into account when determining optimum preparative gel compositions included the degree of resolution obtainable between the individual α

Figure 9. Effect of varying the acrylamide monomer concentration on the resolution of HSV-2 α polypeptides from preparative PAGE. HEp-2 cells were infected with HSV-2 and isotopically labeled with ^{35}S-methionine (25 μCi/ml) from 2 to 6 hrs pi. At 6 hrs pi the whole cell fractions were harvested and prepared for preparative PAGE. The labeled infected cell extracts were then resolved by preparative PAGE using separating gels with varying concentrations of the acrylamide monomer: (A) 5%, (B) 6%, (C) 7%, and (D) 10%. The bars indicate the fractions which were pooled to demonstrate the presence of the particular α polypeptide.

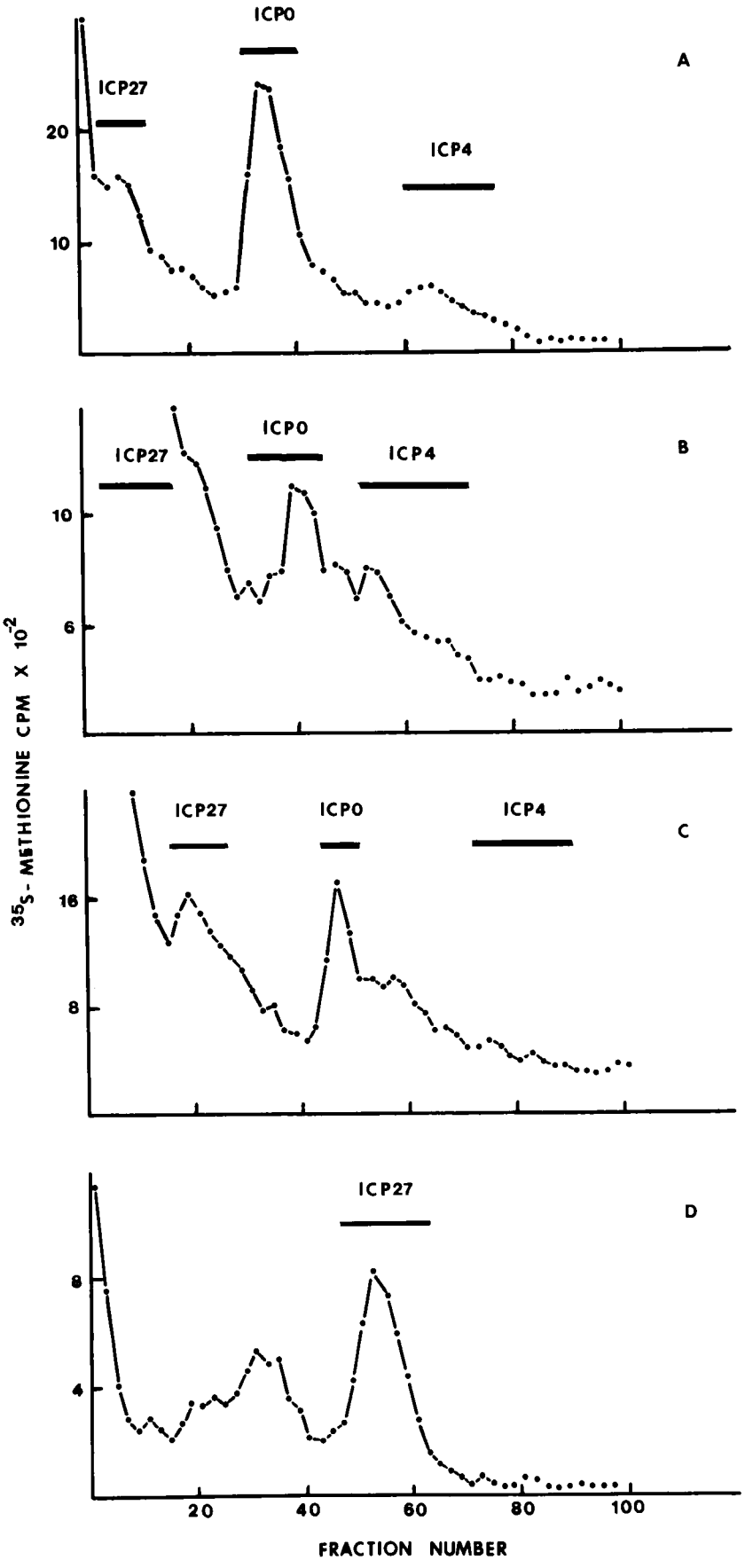
ICP0
A
ICP27
20
ICP4
10
ICP0
B
ICP27
ICP4
10
6
ICP27
ICP0
ICP4
C
16
8
ICP27
D
8
4
20
40
60
80
100
FRACTION NUMBER
35S- METHIONINE CPM X 10-2

Figure 9.

polypeptides and the total volume in which the proteins eluted. Separating gels of 7% acrylamide monomer composition (Figure 9C) were found to yield the best preparative results and were chosen for use in all primary antigen preparations.

Preparative SDS-PAGE purification of α polypeptide immunogens. Subconfluent HEp-2 cells were infected with HSV-2 at a moi of 20-40 pfu/cell and incubated as described in Materials and Methods. Antigens were produced either in the presence or absence of the arginine analogue, canavanine. Antigen produced in the presence of canavanine was isolated from the nuclear fraction of infected cells (Materials and Methods). The nuclear fraction from approximately 1.5 X 10^8 cells was used for each preparative acrylamide (5%) gel run. Antigen preparations were solubilized as described in Materials and Methods. Briefly, the samples were disrupted by ultrasonic vibration and heating at 100°C in the presence of SDS, urea, and β-mercaptoethanol. Antigens from infected cells cultures in the presence of canavanine were obtained from whole cell extracts and solubilized as described above. Because of the increased protein content of the whole cell extract and the diffuse migration characteristics of polypeptides from canavanine treated cultures, fewer cells could be applied to each preparative gel and a higher separating

gel concentration had to be used. Specifically, 8 X 10^7 cells were applied to each run of a 6 or 7% acrylamide separating gel.

A schematic representation of the preparative PAGE purification of HSV-2 α polypeptide immunogens prepared in canavanine inhibited cells is presented in Figure 10. Panel A is an electrophoretic profile of antigens eluted off a 6% preparative gel and demonstrates the separation of all the α polypeptides. An analytical slab gel autoradiogram showing the polypeptides isolated from this preparative gel is shown in the insert in Panel A of Figure 10. Fractions containing the ICP27 polypeptide were collected, concentrated, and re-electrophoresed on a 10%, 60 ml separating gel (Figure 10B). This step sufficiently separated the ICP27 antigen from the contaminating cell actin protein. The fractions containing the desired ICP27 protein were then concentrated and reapplied to a 10% preparative gel. The gel quantity was reduced from 60 ml to 50 ml for this run to allow the collection of the polypeptide in the high resolution region around the 50^{th} fraction (Figure 10D). Fractions collected from the primary preparative PAGE containing ICP0 and ICP4 were reapplied to a second preparative PAGE of 7% acrylamide concentration with a 40 ml volume and 6% acrylamide concentration with a 40 ml volume, respectively (Figures 10E and 10C). These gel concentrations and volumes of separating

Figure 10. Purification of HSV-2 α polypeptides by preparative SDS-PAGE. HEp-2 cells were infected with HSV-2 in the presence of 2.8 mM canavanine and prepared for preparative SDS-PAGE as described in Materials and Methods. Briefly, cells were radioactively labeled with ^{35}S-methionine and solubilized immediately prior to electrophoresis by heating at 100°C in the presence of SDS, urea, and β-mercaptoethanol. Panel A is a representative profile demonstrating the degree of polypeptide separation achieved. Fractions containing the desired polypeptides were pooled and concentrated and electrophoresed on a slab gel PAGE to determine the purity of the fractions collected. An autoradiogram of such an analytical gel is presented in the insert shown in Panel A. Panel B is the elution profile obtained after electrophoresis of the fractions containing ICP27 from Panel A. This elution profile was obtained using a 60 ml separating gel of 10% acrylamide monomer concentration. The fractions containing ICP27, separated from proteins that comigrate in a 6% gel (Panel A), were collected and re-electrophoresed on a 50 ml gel of 10% acrylamide monomer concentration (Panel D). Panel C is the elution profile obtained after electrophoresis of the fractions containing ICP4 which were pooled from the preparative gel shown in Panel A. This profile was obtained using a 40 ml separating gel of 6% acrylamide monomer concentration. Panel E is the elution profile obtained after electrophoresis of the fractions containing ICP0 which were

Figure 10 (Cont'd)

pooled from the preparative gel shown in Panel A. This profile was obtained using a 40 ml separating gel of 7% acryalmide monomer concentration.

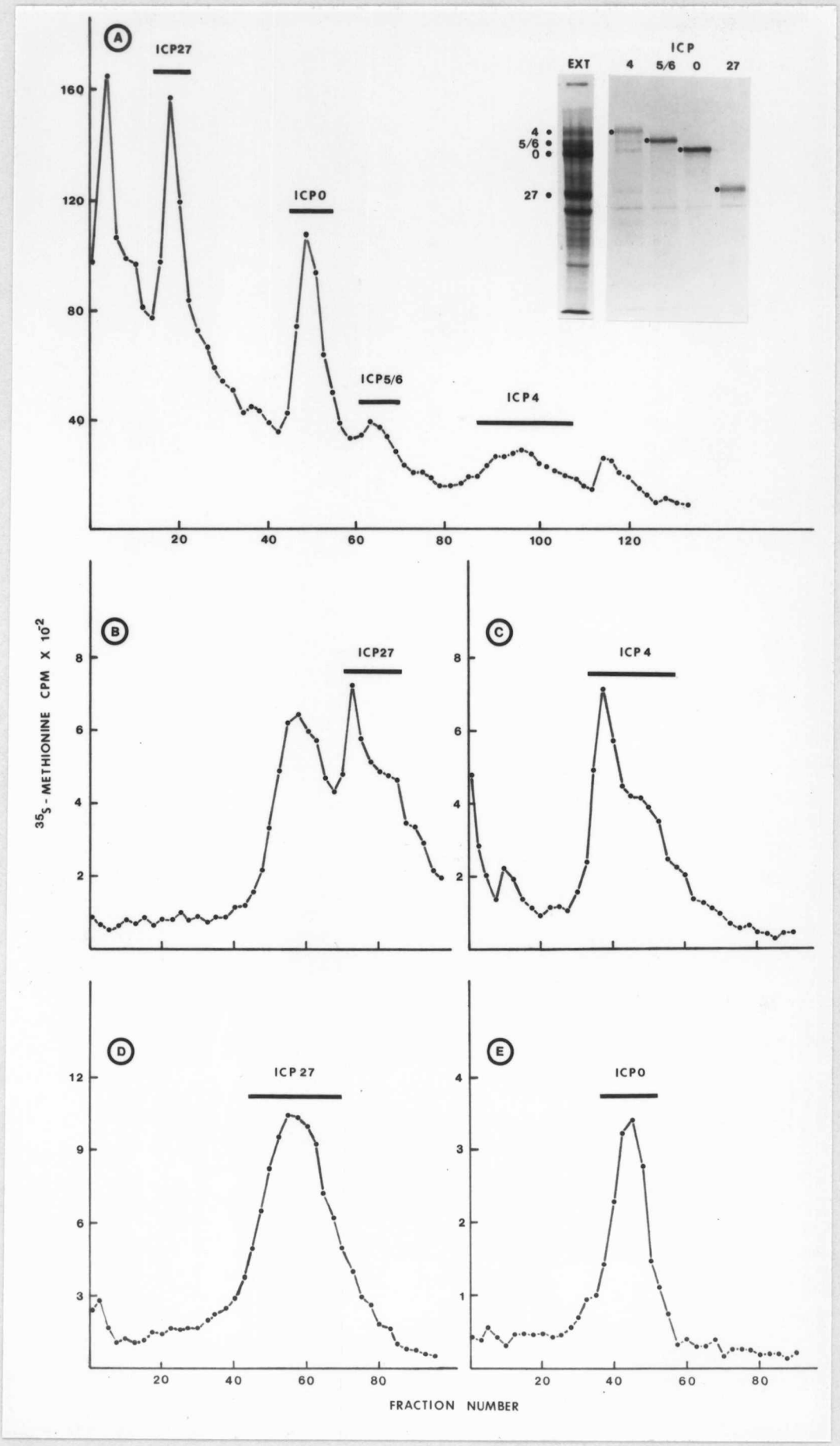
A
ICP27
ICP0
ICP5/6
ICP4
EXT
ICP
4
5/6
0
27
160
120
80
40
20
40
60
80
100
120
B
ICP27
C
ICP4
D
ICP 27
E
ICP0
^{35}S - METHIONINE CPM X 10^{-2}
FRACTION NUMBER

Figure 10.

gel were chosen to facilitate the highest degree of resolution. Final preparations of all immunogens were assayed by analytical slab gel PAGE and the autoradiogram of this analysis demonstrates the purity of the antigen (Figure 11A). Polypeptides extracted from Dounce homogenized HSV-infected cells grown in the absence of the inhibitor were purified in a similar fashion and yielded comparable results (data not shown).

Banding of antigen in polyacrylamide tube gels and quantitation of antigen inocula. The purified α polypeptides obtained from preparative PAGE were concentrated to approximately 1 ml and electrophoresed into cylindrical tube gels as described in Materials and Methods. A representative gel from each antigen preparation was stained with Coomassie brilliant blue and used to indicate the presence of any unlabeled contaminating cellular polypeptides and also as an analytical tool for the quantitation of the antigen banded in the gel. The gels were analysed using a scanning densitometer as described in Materials and Methods. Briefly, stained gels were scanned at 540 nm and densitometric tracings were obtained which showed both the number and density of bands in each gel.

Figure 11B demonstrates the presence of only one detectable protein band in cylindrical gels stained with Coomassie brilliant blue. This technique is not as sensitive as autoradiographic techniques which detect

Figure 11. Purity of HSV-2 α polypeptide antigen preparations. HSV-2 alpha polypeptides isotopically labled with ^{35}S-methionine were produced in HEp-2 cells infected in the presence of 2.8 mM canavanine and purified by preparative SDS-PAGE. The purity of each α polypeptide-containing sample was assayed by autoradiographic analysis of slab gel PAGE of the sample. The purified antigens used to raise antisera against ICP4, ICP0, and ICP27 are presented in Panel A. Panel B demonstrates densitometric analyses of the antigens re-electrophoresed in polyacrylamide tube gels and stained with Coomassie brilliant blue.

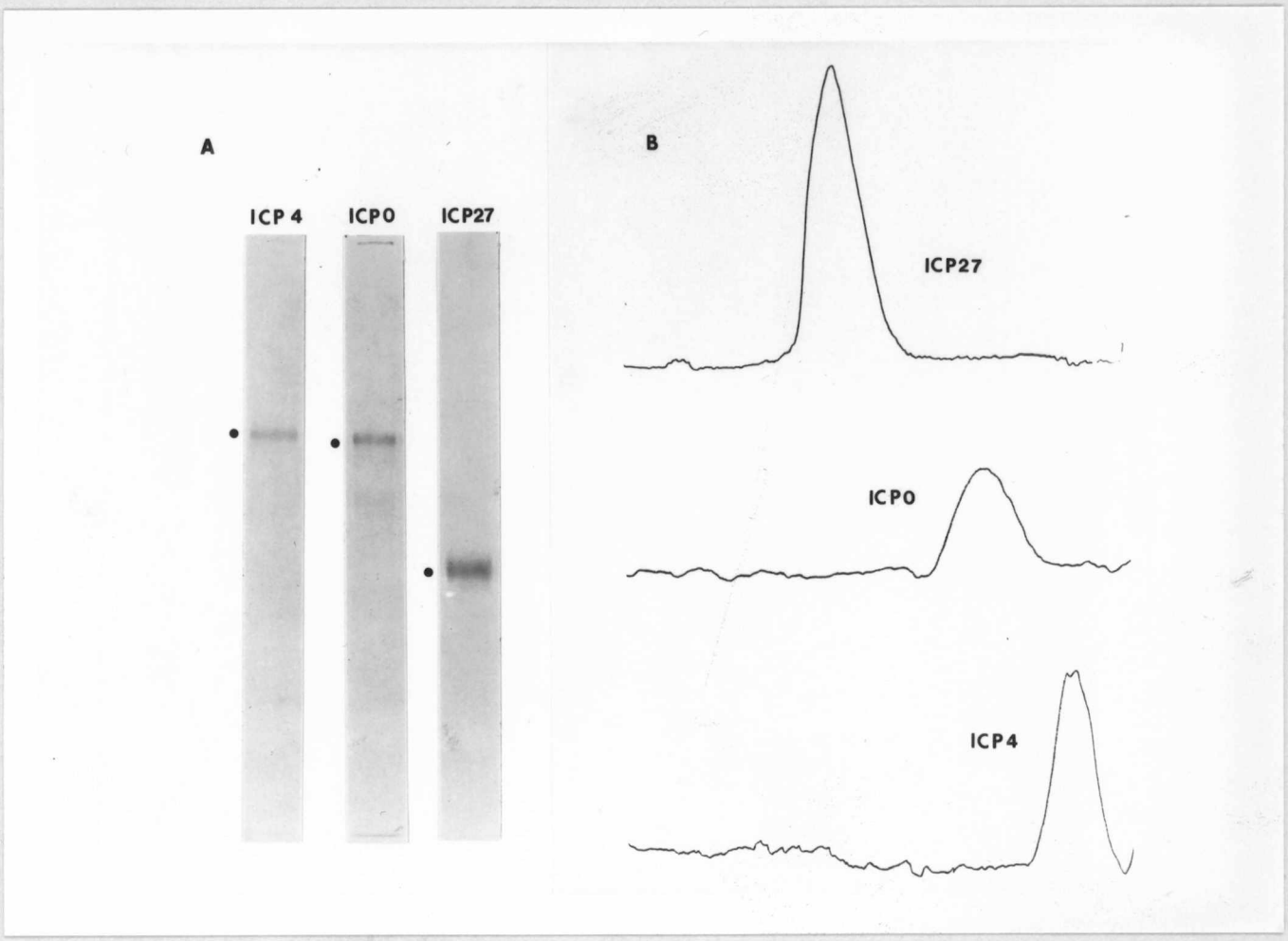

Figure 11.

radioactively labeled proteins, but is a necessary assay in this system. The detection of contaminating host-specified polypeptides by autoradiography is unlikely because of the early inhibition of host-specified polypeptide synthesis. The mere inhibition of host protein synthesis does not rule out the presence of polypeptides made prior to the time of inhibition, however, and the need to detect any possible non-viral contaminants necessitates the use of staining with Coomassie brilliant blue.

An estimation of the amount of the purified antigen in each band was obtained by using a densitometric comparison of the area found beneath the curves corresponding to the purified antigen and the area found beneath the curves corresponding to known concentrations of BSA protein. A semi-logarithmic graph was produced by plotting the peak weight (directly related to the area beneath the curve and determined by weighing on a Metler H30 analytical scale) along the logarithmic ordinate axis and the protein concentration along the abscissa. Figure 12 shows the linear relationship obtained with this determination and also presents the quantitative results obtained. Using the fact that approximately 1 ml of each antigen was originally available in pure form, approximately 100, 70, and 300 µg of protein were used to immunize each rabbit with ICP4, ICP0, and ICP27, respectively.

Figure 12. Densitometric estimation of α polypeptide antigen concentrations. Serial two-fold dilutions of a known concentration of bovine serum albumin (BSA) were electrophoresed into polyacrylamide tube gels and stained with Coomassie brilliant blue. Densitometric tracings of the stained gels were obtained and the curves corresponding to each dilution band were integrated by cutting out and weighing the peaks. Linear regression was used to obtain the standard curve produced by plotting the logarithm of the weight of each peak (in grams) against its corresponding concentration of protein. Densitometric tracings of HSV-2 α polypeptide immunogens were prepared as described and the peak corresponding to each antigen was cut out and weighed. The weights of each peak obtained were plotted on the BSA standard curve and the protein concentration of each sample estimated. The concentrations obtained are presented in a tabular insert.

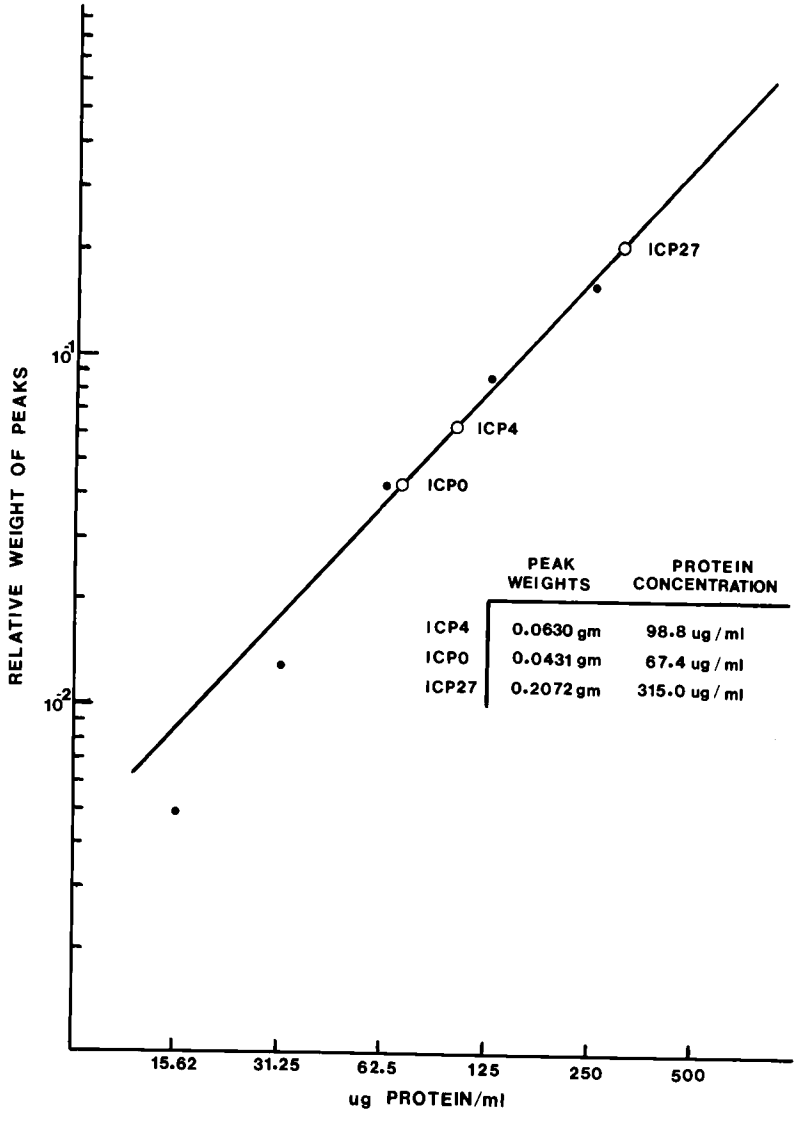
RELATIVE WEIGHT OF PEAKS
10^{-1}
10^{-2}
ICP27
ICP4
ICP0
PEAK WEIGHTS
PROTEIN CONCENTRATION
ICP4 0.0630 gm 98.8 ug / ml
ICP0 0.0431 gm 67.4 ug / ml
ICP27 0.2072 gm 315.0 ug / ml
15.62
31.25
62.5
125
250
500
ug PROTEIN/ml

Figure 12.

Specificity of antisera produced against highly purified HSV-2 α polypeptide antigens. The antisera were characterized by examination of the actual polypeptides precipitated in RIP experiments. This was accomplished by coupling the RIP procedure with polyacrylamide gel electrophoresis. Precipitates obtained were solubilized and electrophoresed in slab gel PAGE. Autoradiograms of these gels were examined to determine the specificity of the antisera. Figure 13 shows an autoradiogram of the immunoprecipitate obtained with anti-ICP4 antisera. Specificity is demonstrated by the immunoprecipitation of ICP4 from the isotopically-labeled cell extract. Anti-ICP0 antiserum was tested in an RIP assay utilizing immediate-early polypeptides labeled with ^{35}S-methionine which were purified by preparative SDS-PAGE and used, along with detergent extracts of whole HSV-2 infected cells, for antigen in the RIP procedure. Antiserum directed against ICP0 was effective in immunoprecipitating only the ICP0 polypeptide, either from the preparative PAGE purified antigens or from the whole cell extracts of HSV-2 infected cells (Figure 14). Immunoprecipitation tests performed using antiserum produced against ICP27 also indicate monospecificity. Figure 15 is an autoradiogram of RIP-PAGE showing the polypeptide immunoprecipitated by anti-ICP27. Antigens used in this assay consisted of

Figure 13. Autoradiogram of immunoprecipitate obtained using anti-ICP4 antiserum. HEp-2 cells used to produce antigen for RIP were infected with HSV-2 in the presence of canavanine and isotopically labeled with ^{35}S-methionine from 2-6 hrs pi. Infected cell antigens were detergent extracted as described in Materials and Methods and the solubilized antigens immunoprecipitated using monospecific antiserum against ICP4. The immunoprecipitates obtained were prepared for analysis by slab gel electrophoresis by boiling for 30 sec in the presence of SDS, urea, and β-mercaptoethanol. Lane A: whole cell extract of HSV-2 infected cells cultured in the presence of canavanine and labeled with ^{35}S-methionine; Lane B: whole cell extract of HSV-2 infected cells cultured in the presence of azetidine and labeled with ^{35}S-methionine; Lane C: immunoprecipitate obtained from solubilized whole cell extract of HSV-2 infected cells cultured in the presence of canavanine. The positions of the three α polypeptides are indicated.

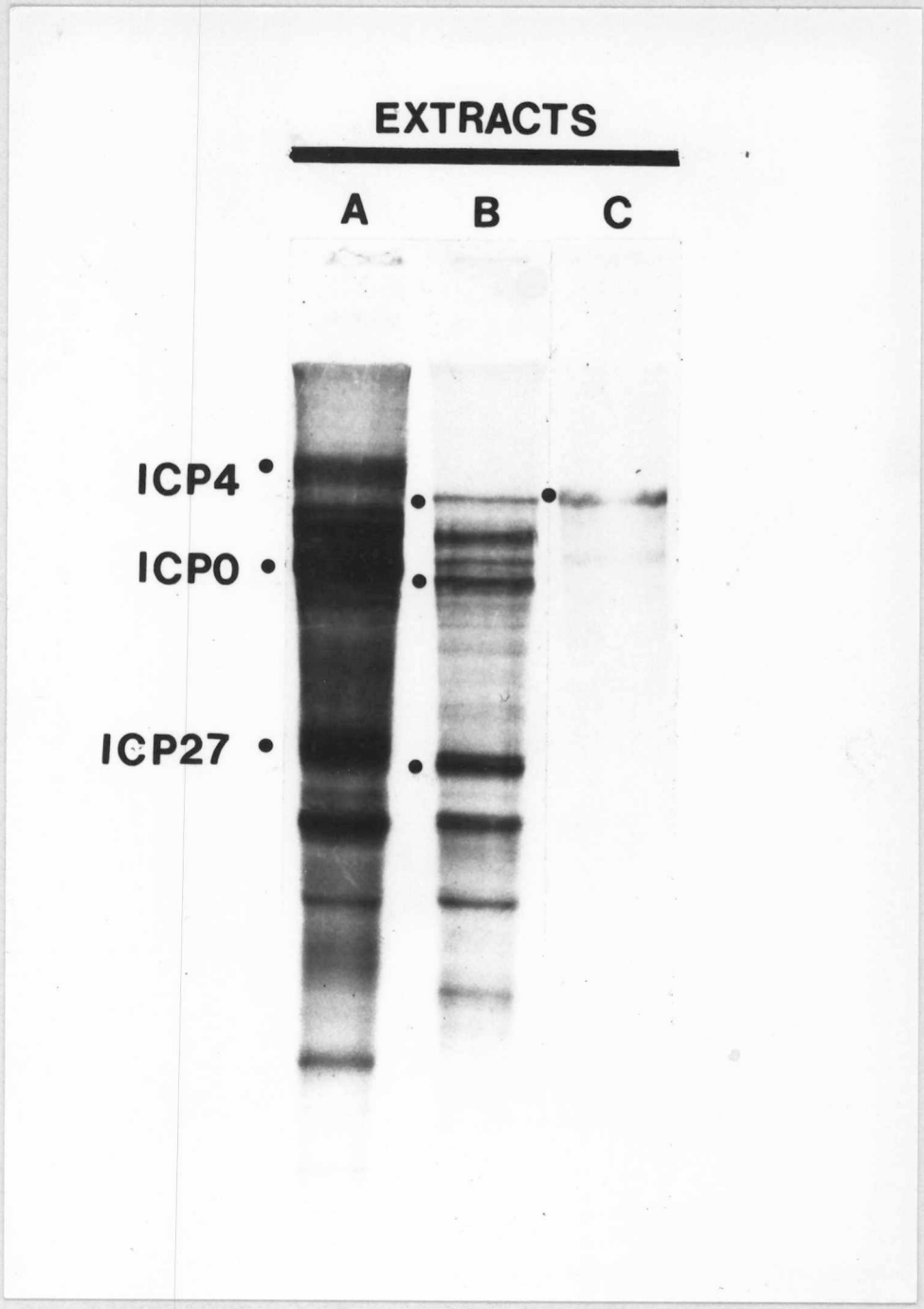

Figure 13.

Figure 14. Autoradiogram of immunoprecipitate obtained using anti-ICP0 antiserum. HEp-2 cells used to produce antigen for the immunoprecipitation were infected with HSV-2 in the presence or absence of canavanine and isotopically labeled with ^{35}S-methionine. Antigens produced in the absence of canavanine were resolved and purified by preparative PAGE and the purified proteins used for RIP antigen. While cell extracts of HSV-2 infected cells cultured in the presence of canavanine were also detergent solubilized as described in Materials and Methods for use in RIP. Immunoprecipitates obtained using antiserum produced against ICP0 were prepared for analysis by slab gel electrophoresis as described. Extracts (EXT) of cells infected with HSV-2 and cultured in the presence of canavanine (CAN) or azetidine (AZE) are included to indicate the positions of the RIP antigens used. Immunoprecipitation was performed using anti-ICP0 antiserum against preparative PAGE purified infected cell polypeptides (4, 5/6, 0, 27, and actin - ACT) or against a whole cell extract of HSV-2 infected cells cultured in the presence of canavanine (CAN). The positions of the five purified antigens used are indicated.

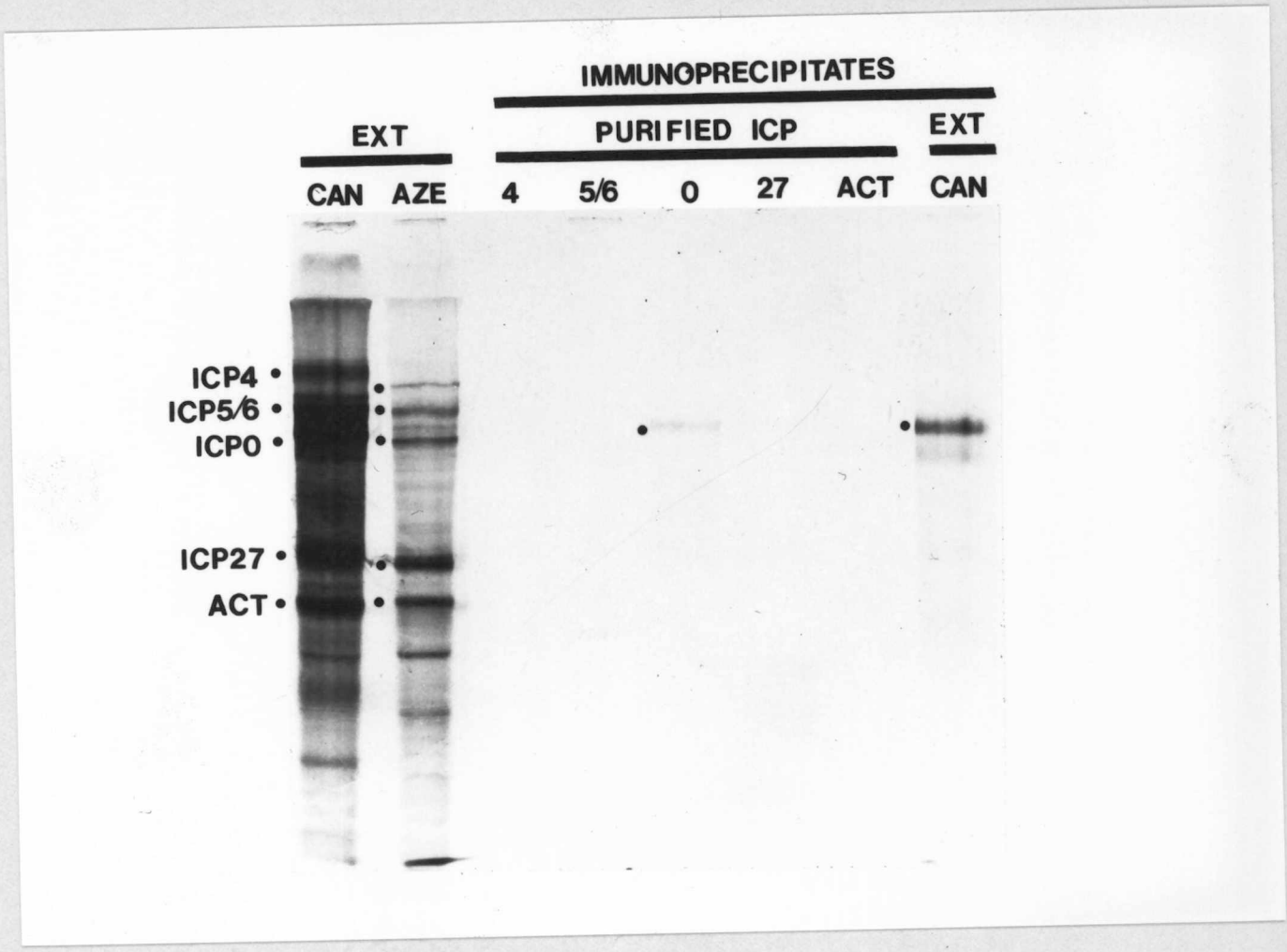
IMMUNOPRECIPITATES
EXT
PURIFIED ICP
EXT
CAN
AZE
4
5/6
0
27
ACT
CAN
ICP4
ICP5/6
ICP0
ICP27
ACT

Figure 14.

Figure 15. Autoradiogram of immunoprecipitate obtained using anti-ICP27 antiserum. HEp-2 cells used to produce antigen for the immunoprecipitation were infected with HSV-2 in the presence or absence of canavanine and isotopically labeled with ^{35}S-methionine. Antigens produced in the absence of canavanine were resolved and purified by preparative PAGE and the purified proteins used for antigen in the RIP procedure. Whole cell extracts of HSV-2 infected cells cultured in the presence of canavanine were also detergent solubilized as described in Materials and Methods for use in RIP. Immunoprecipitates obtained using antiserum produced against ICP27 were prepared for analysis by slab gel electrophoresis as described. Extracts (EXT) of cells infected with HSV-2 and cultured in the presence of canavanine (CAN) or azetidine (AZE) are included to indicate the positions of the RIP antigens used. Immunoprecipitation was performed using anti-ICP27 antiserum against preparative PAGE purified infected cell polypeptides (4, 5/6, 0, 27, and actin - ACT) or against a whole cell extract of HSV-2 infected cells cultured in the presence of canavanine (CAN). The positions of the five purified antigens used are indicated.

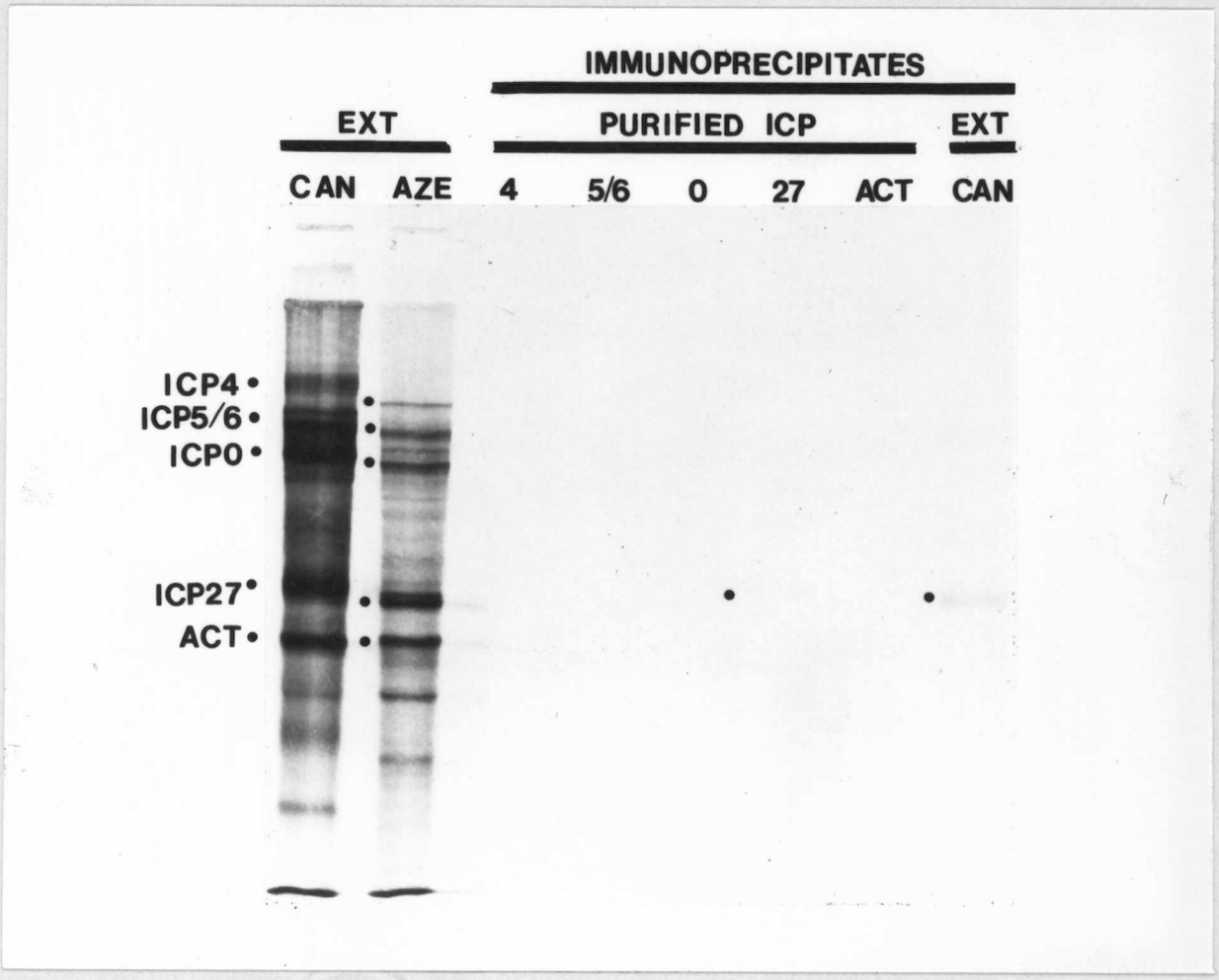
IMMUNOPRECIPITATES
EXT
PURIFIED ICP
EXT
CAN
AZE
4
5/6
0
27
ACT
CAN
ICP4
ICP5/6
ICP0
ICP27
ACT

Figure 15.

both whole cell extracts and purified antigens as described above.

Analysis of α Polypeptide Expression in Cells Infected with HSV-2

Intracellular location and time of appearance of HSV-2 infected cell α polypeptides. The α polypeptides produced in HSV-2 infected cells can be shown by PAGE of cells isotopically labeled at various times post infection to be synthesized very early in the replication cycle (Figures 3, 7, and 8, pages 53, 65, and 67 respectively). The synthesis of the polypeptides in this group is inhibited later in the infection, at which time the production of members of the group becomes undetectable. Because the polypeptides which are labeled during a one hour pulse period early in the infection are still present in cells harvested up to 24 hrs pi, it was interesting to investigate the intracellular expression of the polypeptides at various times post infection. Indirect immunofluorescence using monospecific antisera raised in rabbits to each of the individual α polypeptides was used for this investigation.

Vero cells were seeded onto glass coverslips, infected with HSV-2 and incubated at 37°C as described in Materials and Methods. The coverslips were removed from the incubator at appropriate times and stained with

monospecific antisera. Cultures treated in this manner and stained with anti-ICP4 are shown in Figure 16. Specific immunofluorescence can be seen in the cytoplasmic portion of infected cells by 2 hrs pi. Cells harvested at subsequent time points show a decreased cytoplasmic staining and an apparent migration of ICP4 to the nuclei of infected cells. The immunofluorescence observed with anti-ICP4 antiserum exhibited a distinctly granular intranuclear appearance. The antigen appeared to accumulate in the nucleus and the fluorescent granules became brighter at later times post infection. Uninfected Vero cells did not fluoresce when stained with this antiserum, nor did HSV-2 infected Vero cells stained with pre-immune rabbit serum.

Immunofluorescent analysis of HSV-2 infected Vero cells stained with anti-ICP0 was performed as described above and representative photomicrographs are shown in Figure 17. Infected cells showed a nuclear fluorescence similar to that described for ICP4. The time of appearance of ICP0 was found to be different from that of ICP4. A granular nuclear fluorescence is obvious in cells harvested as early as 1 hr pi. The intensity of this granular fluorescence increases through 4 hrs pi, at which time the granules appear to coalesce and marginate at the interior of the infected cells nuclei. Cells harvested at 6 hrs post infection show a marginated fluorescence of decreasing intensity. Specific fluorescence using the anti-ICP0

Figure 16. Indirect immunofluorescence of HSV-2 infected cells stained at various times post infection with anti-ICP4. Vero cells cultured on glass coverslips were infected with HSV-2 and incubated at 37^{o}C. Cultures were harvested at various times pi and prepared for immunofluorescent analysis as described in Materials and Methods. Panel A: uninfected MRC-5 cells stained with anti-ICP4; Panels B, C, D, E, F, G, and H: HSV-2 infected MRC-5 cells harvested and stained with anti-ICP4 antiserum at 2, 3, 4, 5, 6, 8, and 10 hrs pi, respectively; Panel I: HSV-2 infected MRC-5 cells stained with preimmune rabbit serum.

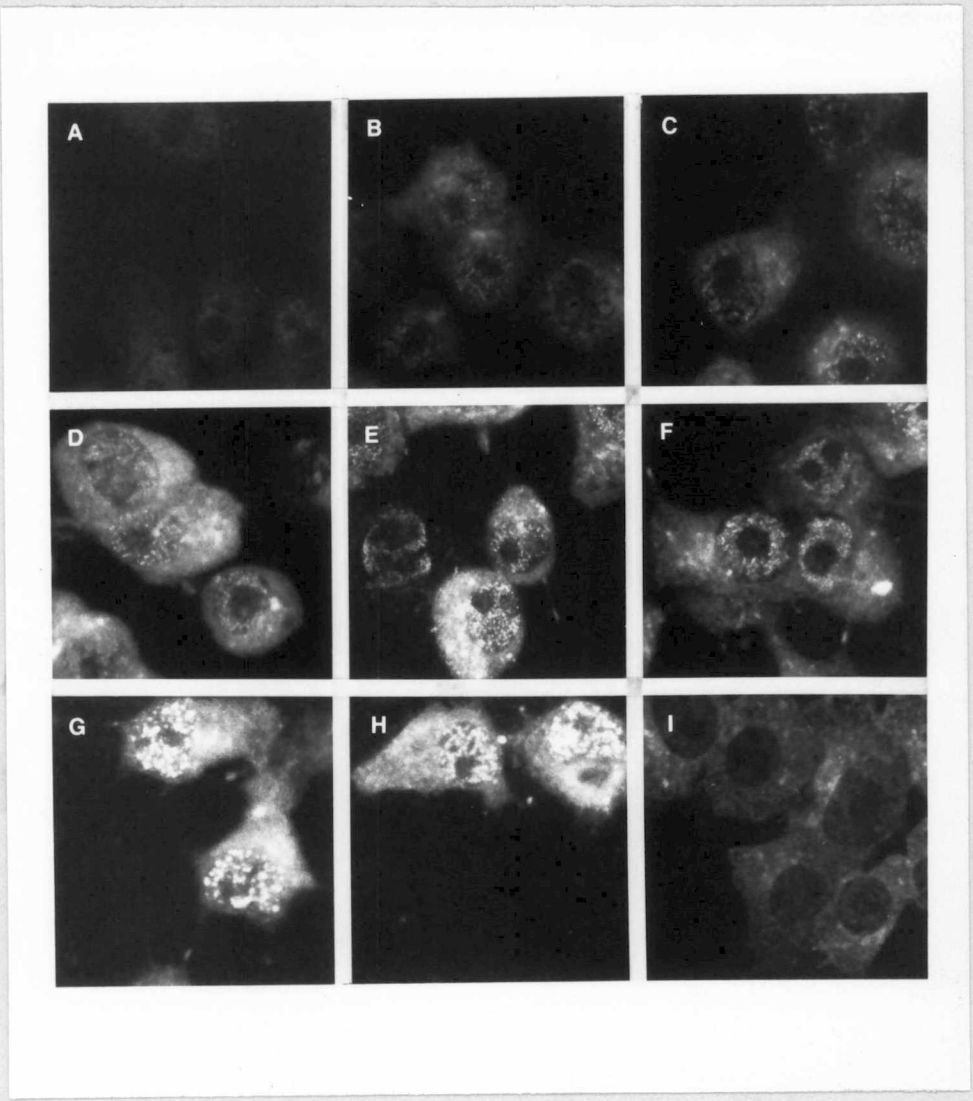

Figure 16.

Figure 17. Indirect immunoflorescence of HSV-2 infected cells stained at various times post infection with anti-ICP0 antiserum. MRC-5 cells cultured on glass coverslips were infected with HSV-2 and indubated at 37°C. Cultures were harvested at various times pi and prepared for immunofluorescent analysis as described in Materials and Methods. Panel A: uninfected MRC-5 cells stained with anti-ICP0 antiserum; Panels B, C, D, E, F, and G: HSV-2 infected MRC-5 cells harvested and stained with anti-ICP0 at 1, 2, 3, 4, 5, and 6 hrs pi, respectively; Panel H: HSV-2 infected MRC-5 cells stained with pre-immune rabbit serum.

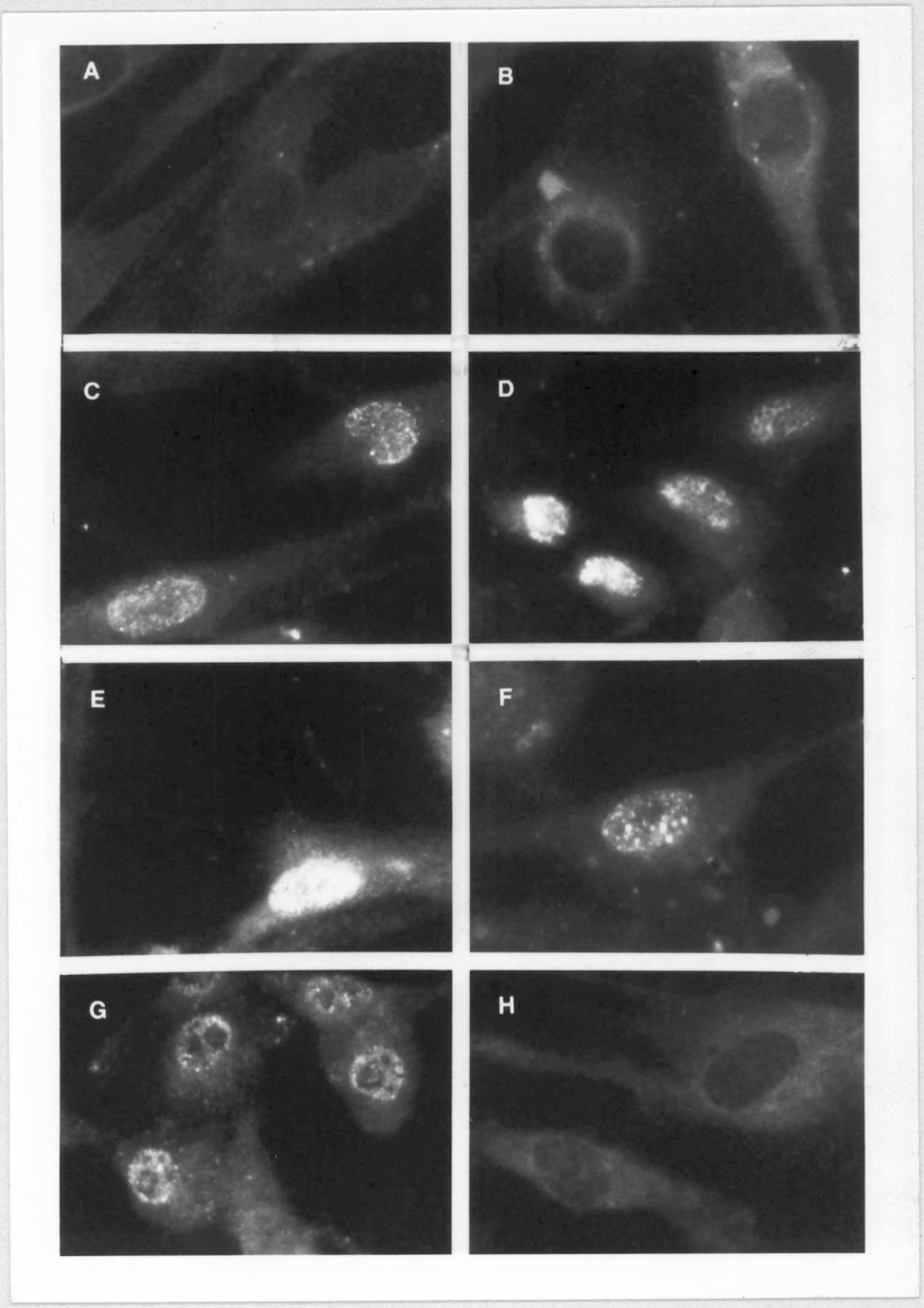

Figure 17.

antiserum could not be detected in cells harvested at later times post infection.

HSV-2 infected Vero cells stained with anti-ICP27 antiserum and examined by indirect immunofluorescence are shown in Figure 18. This antigen exhibited a cytoplasmic fluorescence at all times examined post infection. Specific cytoplasmic fluorescence seen in infected cells stained with this antiserum typically displayed a granular appearance appearing first in the perinuclear area. ICP27 is the only α polypeptide which shows a cytoplasmic location with the exclusion of any nuclear involvement.

The time of appearance of HSV-2 specific polypeptides produced in HSV-2 infected Vero cells as examined by immunofluorescence is demonstrated in Figure 19. The antiserum used in this assay was the positive control antiserum produced against all the HSV-2 specific infected cell antigens produced in RK-13 cells (see Materials and Methods). The antiserum was diluted 1:20 in PBS for use in this assay. A bright fluorescence, seen primarily in the cytoplasmic area of these cells, is seen to appear early in the infection and increase rapidly in intensity.

Reactivity of the HSV-2 α polypeptide antisera with HSV-1 and HSV-2 infected cells. Indirect immunofluorescence using the monospecific antisera against the

Figure 18. Indirect immunofluorescence of HSV-2 infected cells stained at various times post infection with anti-ICP27 antiserum. Vero cells cultures on glass cloverslips were infected with HSV-2 and incubated at 37^{o}C. Cultures were harvested at various times pi and prepared for immunofluorescent analysis as described in Materials and Methods. Panel A: uninfected Vero cells stained with anti-ICP27 antiserum; Panel B, C, D, E, and F: HSV-2 infected Vero cells harvested and stained with anti-ICP27 antiserum at 2, 4, 6, 8, and 10 hrs pi, respectively.

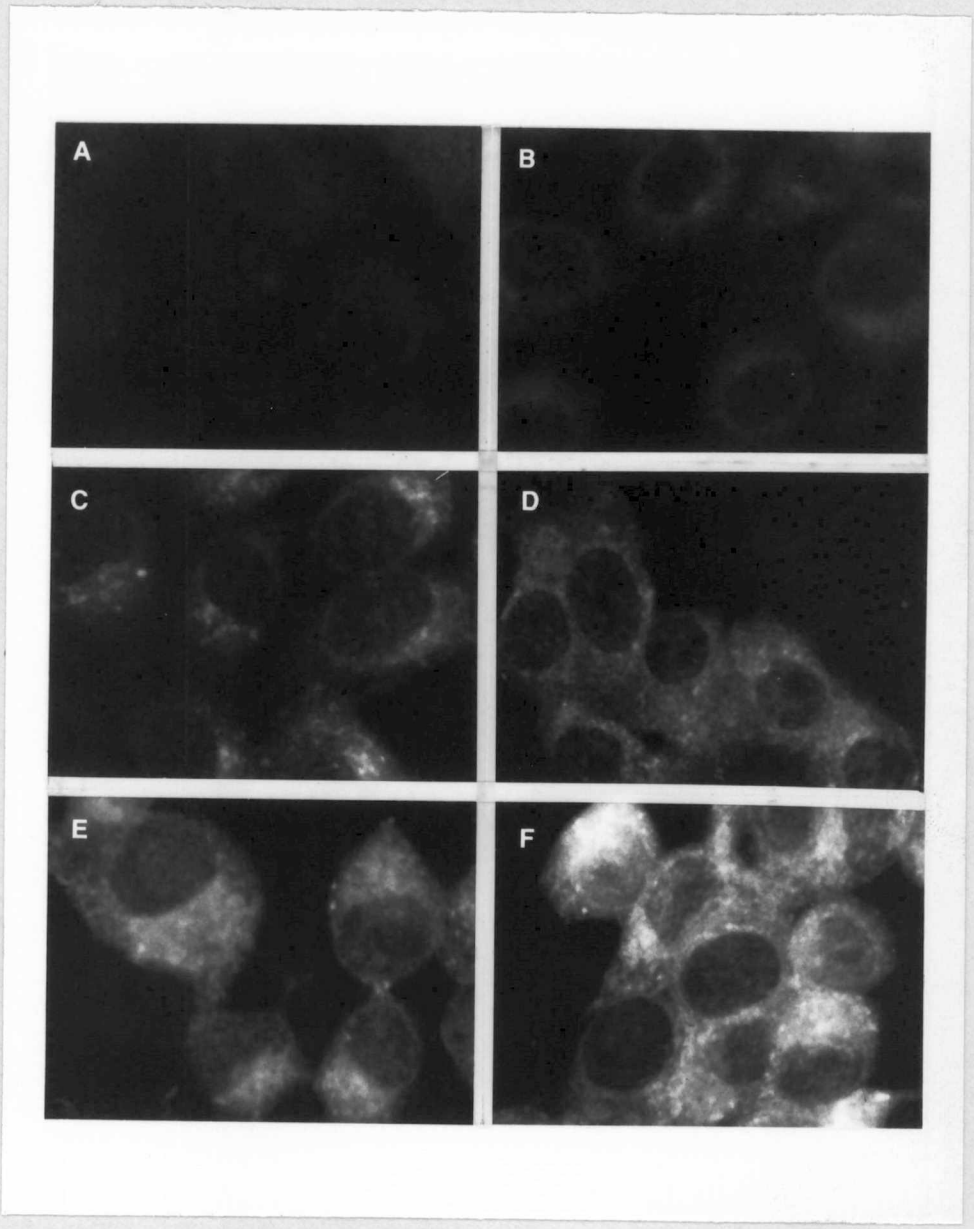

Figure 18.

Figure 19. Indirect immunofluorescence of HSV-2 infected cells stained at various times post infection with anti-HSV-2 antiserum. Vero cells cultured on glass coverslips were infected with HSV-2 and incubated at 37°C. Cultures were harvested at various times pi and prepared for immunofluorescent analysis as described in Materials and Methods. Panel A: uninfected Vero cells stained with anti-HSV-2 antiserum; Panel B, C, D, E, and F: HSV-2 infected Vero cells harvested and stained with anti-HSV-2 antiserum at 2, 4, 6, 8, and 10 hrs pi, respectively.

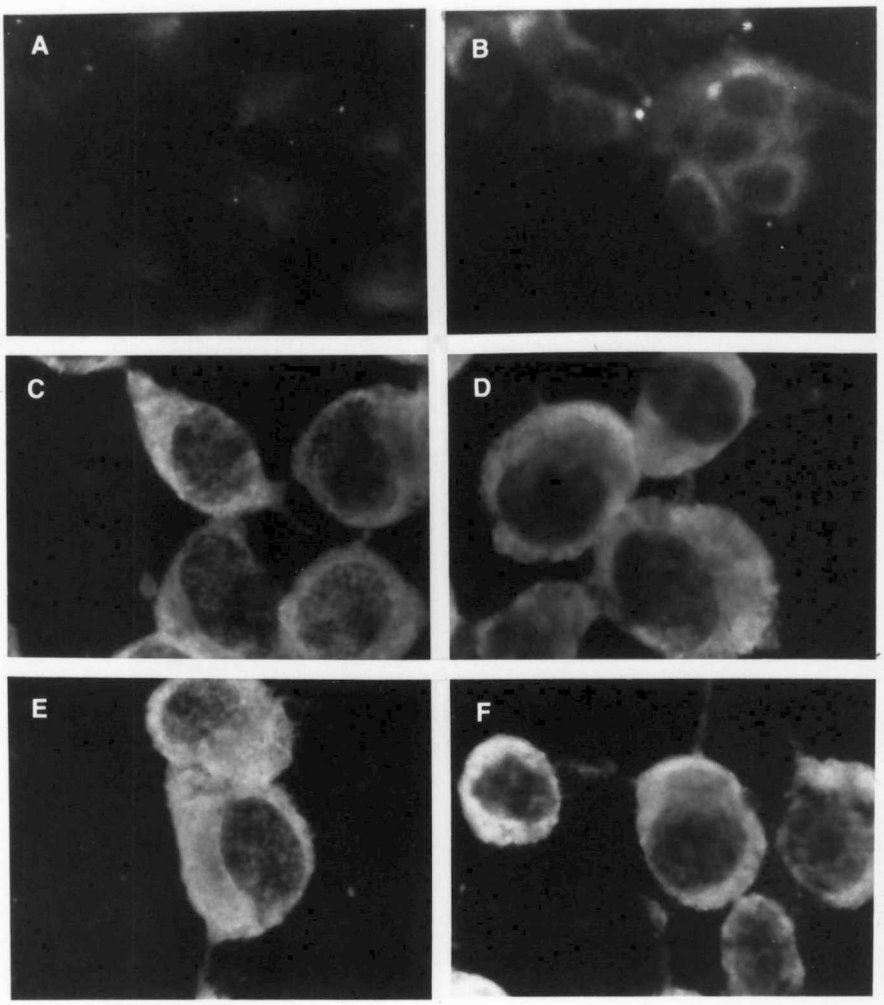

Figure 19.

individually purified HSV-2 α polypeptides was used to determine the cross-reactivity shown by analogous antigens in HSV-1 and HSV-2 infected cells. Coverslips were seeded with Vero or human embryonic lung cells and infected with either HSV-1 or HSV-2. Cultures were harvested at various times post infection and stained with anti-α-polypeptide antisera as described in Materials and Methods.

Human embryonic lung cells were used to determine the type specificity of the ICP4 α polypeptide. Using antiserum produced against the ICP4 produced in HSV-1 infected cells (HSV-1 ICP4), our laboratory has previously presented evidence showing the polypeptide to be type specific. Figure 20 shows immunofluorescence patterns produced in cells stained with the anti-HSV-1 ICP4 antiserum or the anti-HSV-2 ICP4 antiserum produced in this study. HSV-1 ICP4 antiserum shows the type specific nuclear fluorescence exhibited in HSV-1 infected cells (Panel D) but not in HSV-2 infected cells (Panel C). The anti-HSV-2 ICP4 antiserum produced against the HSV-2 α polypeptide, however, reacted positively to both the HSV-1 (Panel B) and HSV-2 (Panel A) infected cells. Thus, the ICP4 α polypeptide produced in HSV-2 infected cells exhibits a non-reciprocal cross-reactivity with HSV-1 infected cells.

Figure 20. Reactivity of anti-ICP4 antiserum with HSV-1 or HSV-2 infected cells. MRC-5 cells cultured on glass coverslips were infected with HSV-1 or HSV-2 and incubated at 37°C. Cultures were harvested at various times post infection and prepared for immunofluorescent analysis as described in Materials and Methods. Panel A: HSV-1 infected cells stained with anti-HSV-2 ICP4; Panel B: HSV-2 infected cells stained with anti-HSV-2 ICP4; Panel C: HSV-1 infected cells stained with anti-HSV-1 ICP4 (ICP4 α polypeptide produced in HSV-1 infected cells); Panel D: HSV-2 infected cells stained with anti-HSV-1 ICP4; Panel E and F: uninfected cells stained with anti-HSV-2 ICP4 and anti-HSV-1 ICP4 antisera, respectively.

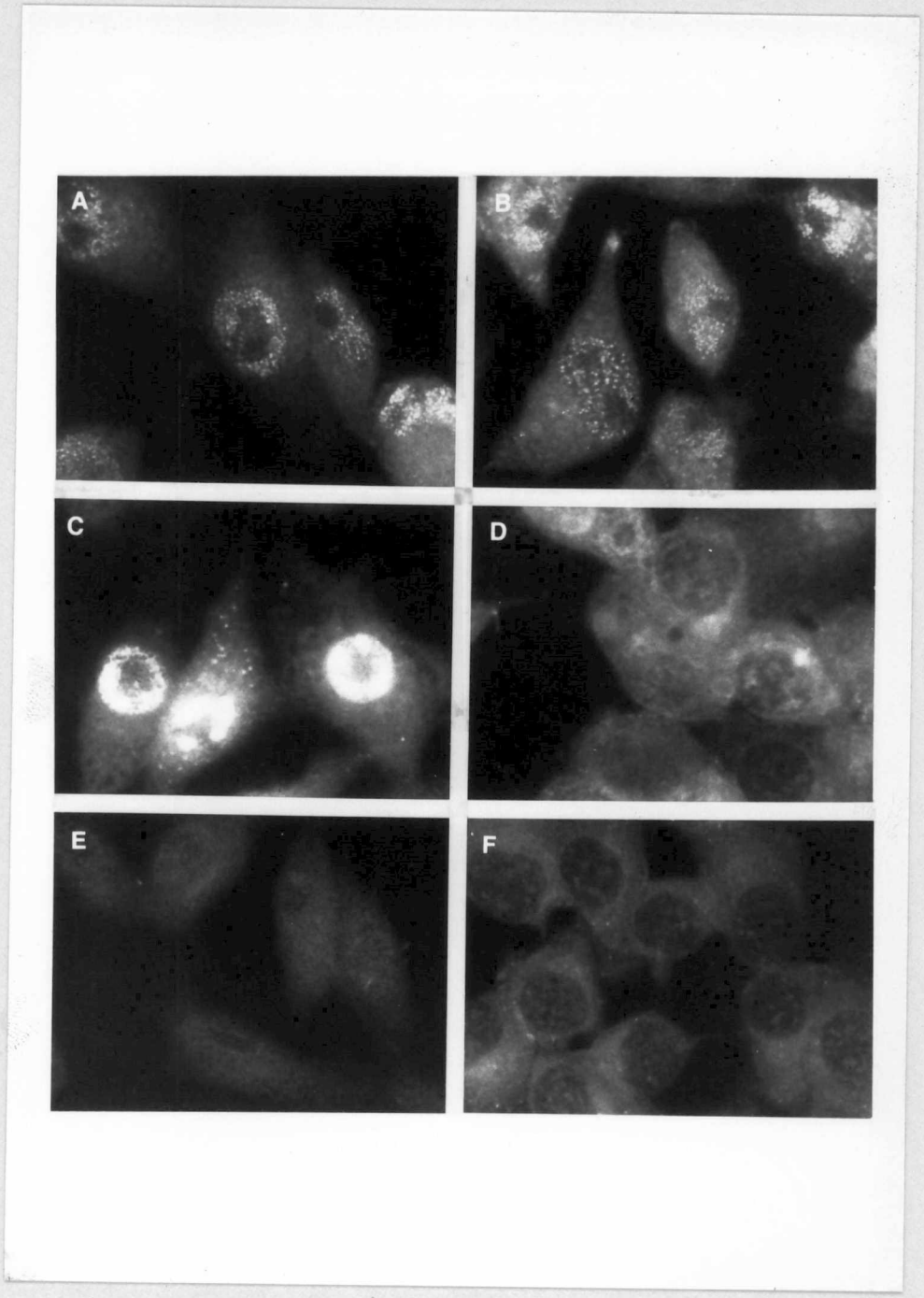

Figure 20.

Anti-ICP0 antiserum produced against the α polypeptide purified from HSV-2 infected cells was used to stain either HSV-1 or HSV-2 infected human embryonic lung cells. The antiserum produced a bright nuclear fluorescence in cells infected with HSV-2 (Figure 21, Panel B) but did not stain cells infected with the HSV-1 (Figure 21, Panel D).

ICP27 is shown in Figure 22 to be a type common polypeptide. The specific cytoplasmic fluorescence produced in cells infected with either the HSV-1 or HSV-2 presents a granular appearance starting in the perinuclear area and spreading throughout the cytoplasm. The staining pattern produced in the HSV-1 infected cells shows a more granular pattern than that seen in the HSV-2 infected cells.

Effects of amino acid analogues on the synthesis and intracellular location of HSV-2 infected cell α polypeptides. Cultures of Vero cells were grown on glass coverslips and infected with HSV-2 in the presence of either 2.8 mM canavanine or 4 mM azetidine as previously described. The cultures were havested with anti-α-polypeptide antisera to study the properties of α polypeptides produced in the presence of amino acid analogues.

Cultures infected with HSV-2 in the presence of canavanine were stained with anti-ICP4 antiserum and

Figure 21. Reactivity of anti-ICP0 antiserum with HSV-1 or HSV-2 infected cells. MRC-5 cells cultured on glass coverslips were infected with HSV-1 or HSV-2 and incubated at 37°C. Cultures were harvested at various times post infection and prepared for immunofluorescent analysis as described in Materials and Methods. Panel A: uninfected cells stained with anti-ICP0; Panel B: HSV-2 infected cells stained with anti-ICP0; Panel C: HSV-2 infected cells stained with pre-immune rabbit serum; Panel D: HSV-1 infected cells stained with anti-ICP0 antiserum.

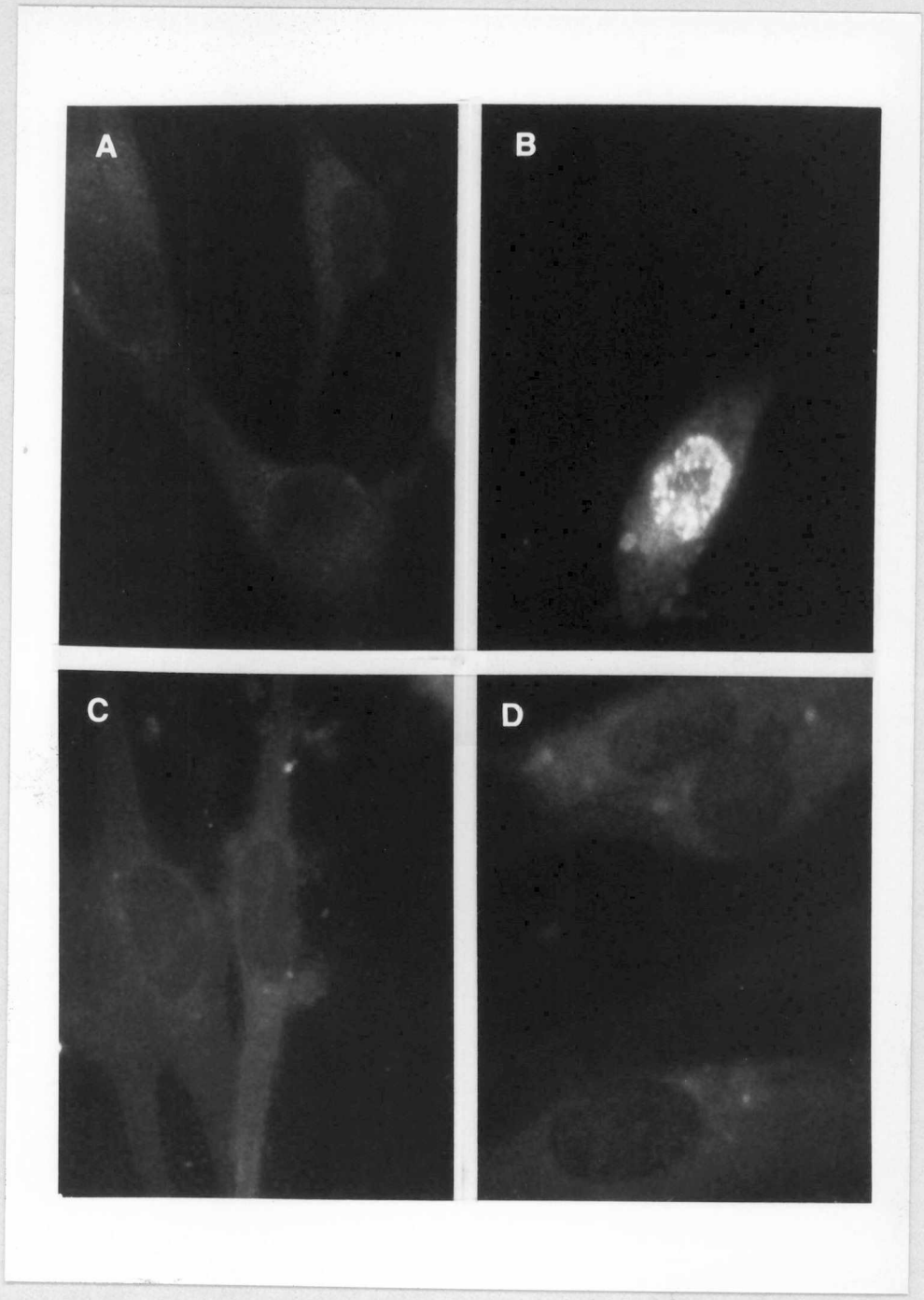

Figure 21.

Figure 22. Reactivity of anti-ICP27 antiserum with HSV-1 or HSV-2 infected cells. Vero cells cultured on glass coverslips were infected with HSV-1 or HSV-2 and incubated at 37°C. Cultures were harvested at various times post infection and prepared for immunofluorescent analysis as described in Materials and Methods. Panel A: uninfected Vero cells stained with anti-ICP27; Panel B: HSV-2 infected Vero cells stained with anti-ICP27; Panel C: HSV-2 infected Vero cells stained with pre-immune rabbit serum; Panel D: HSV-1 infected Vero cells stained with anti-ICP27.

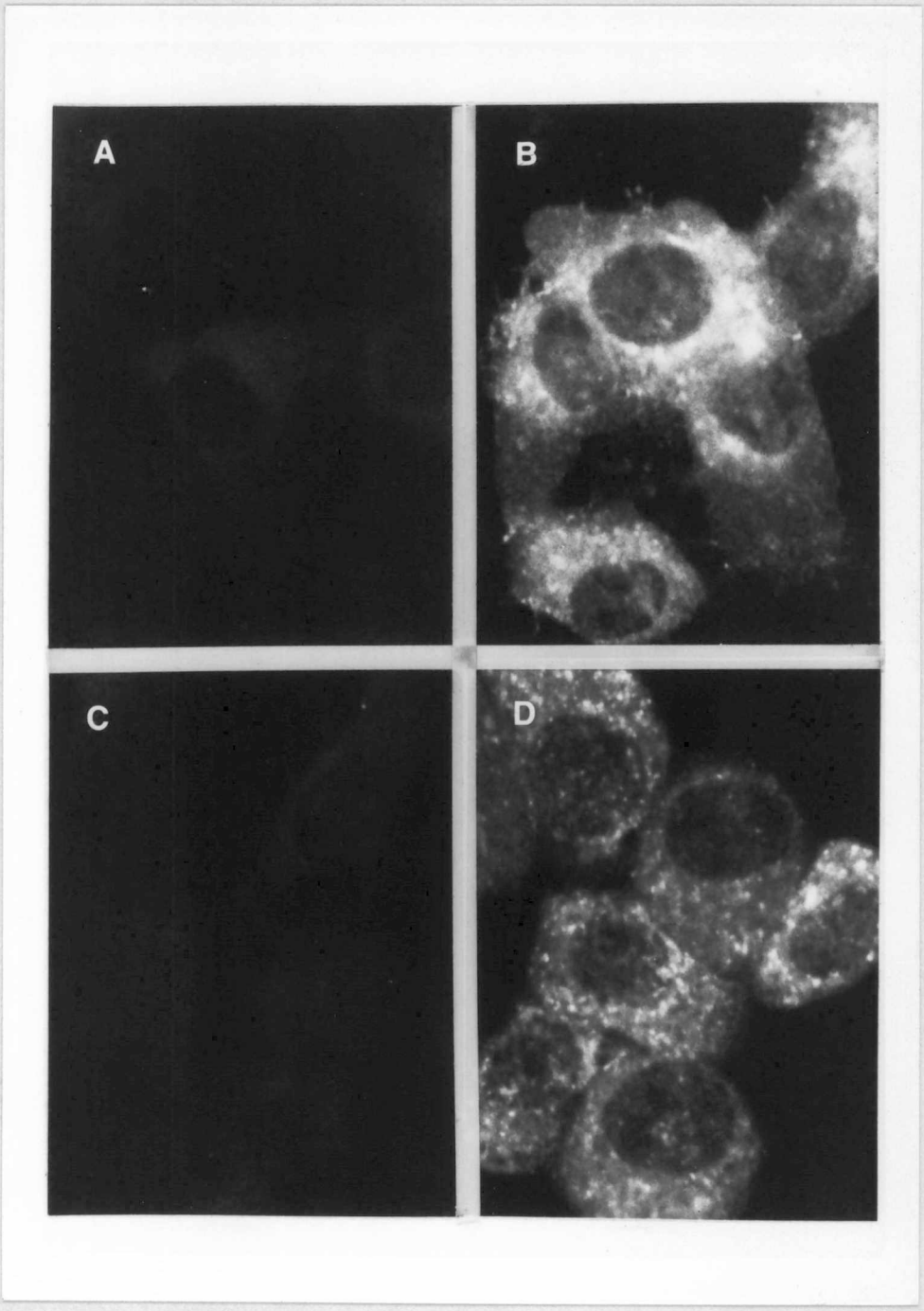

Figure 22.

photomicrographs of the immunofluorescence patterns observed at various times post infection are presented in Figure 23. The migration of ICP4 to the nucleus of infected cells appears to occur as in cells grown in the absence of canavanine; however, it is observed to be maintained at later times pi than in cultures infected in the absence of canavanine. Photomicrographs of HSV-2 infected cells grown in the presence of azetidine and stained for immunofluorescence using anti-ICP4 antiserum are shown in Figure 24. The intracellular migration pattern of ICP4 is shown to be greatly affected. The antigen can be detected only in the cytoplasm of infected cells assayed at various times post infection, and never in the nucleus.

HSV-2 infected cells cultured in the presence of canavanine and stained with anti-ICP0 antiserum produce immunofluorescence patterns shown in Figure 25. There is no detectable difference in the kinetics of synthesis or nuclear location of the antigen produced in cells grown in the presence or absence of canavanine. ICP0 appears very early in infection as a granular nuclear fluorescence and reaches its maximum intensity at 4 to 5 hrs pi. The antigen then fades from the nucleus in a manner analogous to that observed in cells infected in the absence of canavanine. Infected cells cultured in the presence of azetidine also demonstrate the usual early

Figure 23. Anti-ICP4 immunofluorescence obtained in HSV-2 infected cells cultured in the presence of canavanine. Vero cells cultured on glass coverslips and incubated in the presence of canavanine were infected with HSV-2 and harvested at various times post infection for immunofluorescent analysis. Panel A: uninfected cells cultured in the presence of canavanine and stained with anti-ICP4 antiserum; Panel B, C, D, E, and F: HSV-2 infected cells cultured in the presence of canavanine and stained with anti-ICP4 antiserum at 2, 4, 6, 8, and 10 hrs pi, respectively.

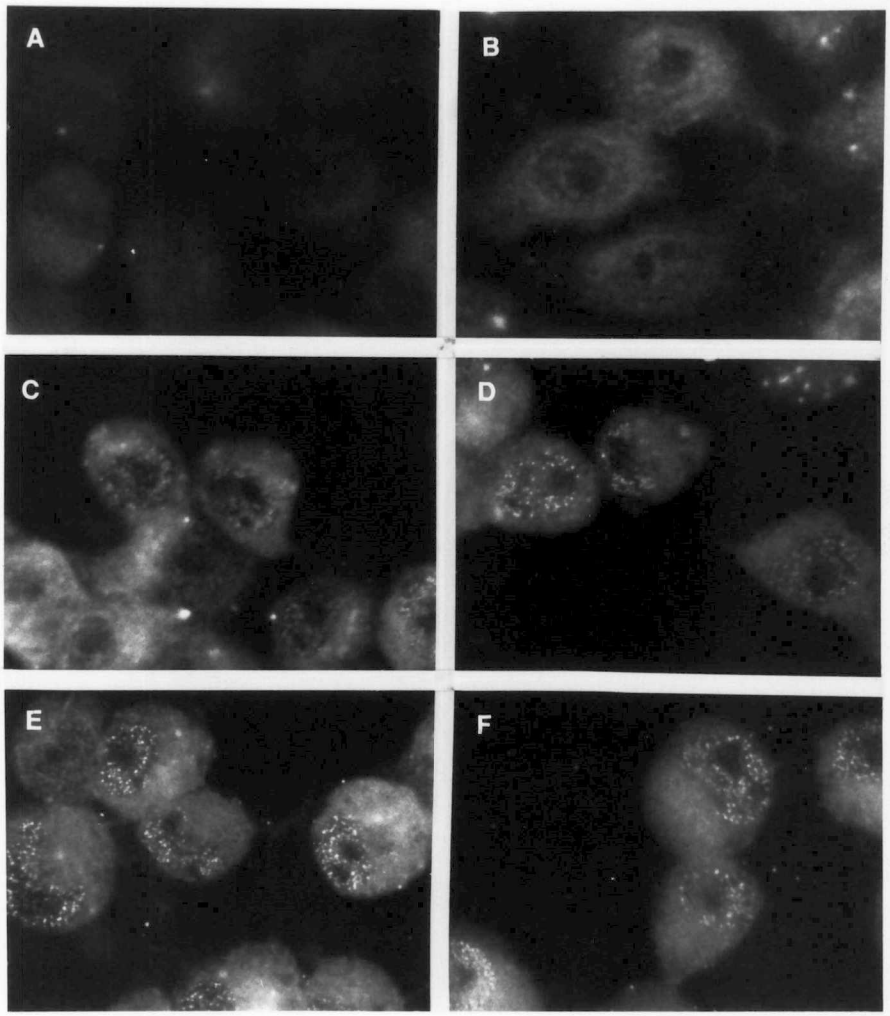

Figure 23.

Figure 24. Anti-ICP4 immunofluorescence obtained in HSV-2 infected cells cultured in the presence of azetidine. Vero cells cultured on glass coverslips and incubated in the presence of azetidine were infected with HSV-2 and harvested at various times post infection for immunofluorescent analysis. Panel A: uninfected cells cultured in the presence of azetidine and stained with anti-ICP4 antiserum; Panels B, C, D, E, and F: HSV-2 infected cells cultured in the presence of azetidine and stained with anti-ICP4 antiserum at 2, 4, 6, 8, and 10 hrs pi, respectively.

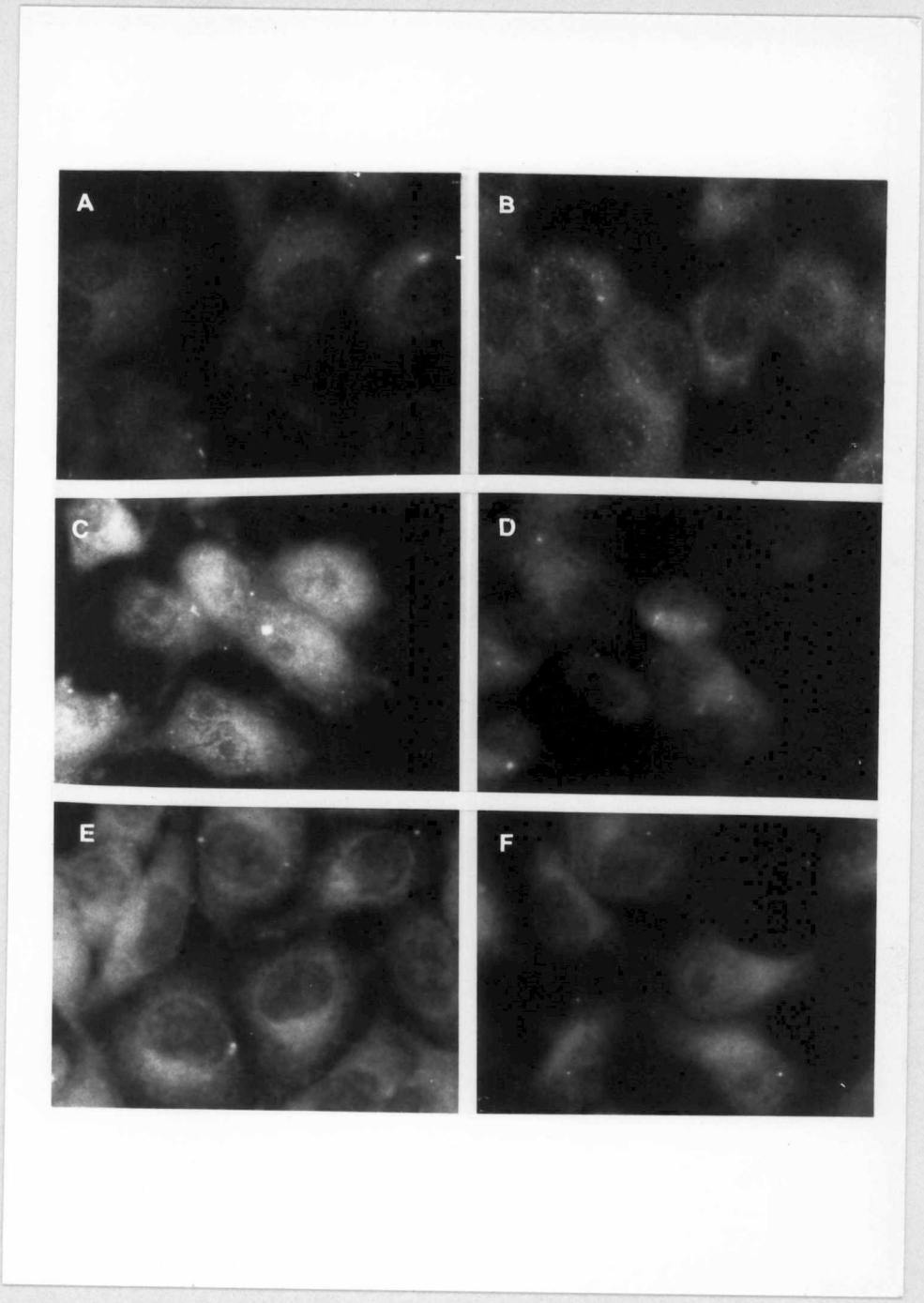

Figure 24.

Figure 25. Anti-ICP0 immunofluorescence obtained in HSV-2 infected cells cultured in the presence of canavanine. Vero cells cultured on glass coverslips and incubated in the presence of canavanine were infected with HSV-2 and harvested at various times post infection for immunofluorescent analysis. Panel A: uninfected cells cultured in the presence of canavanine and stained with anti-ICP0 antiserum; Panel B, C, D, E, F, and G: HSV-2 infected cells cultured in the presence of canavanine and stained with anti-ICP0 antiserum at 1, 2, 4, 6, 8, and 10 hrs post infection, respectively; Panel H: HSV-2 infected cells cultured in the presence of canavanine and stained with pre-immune rabbit serum.

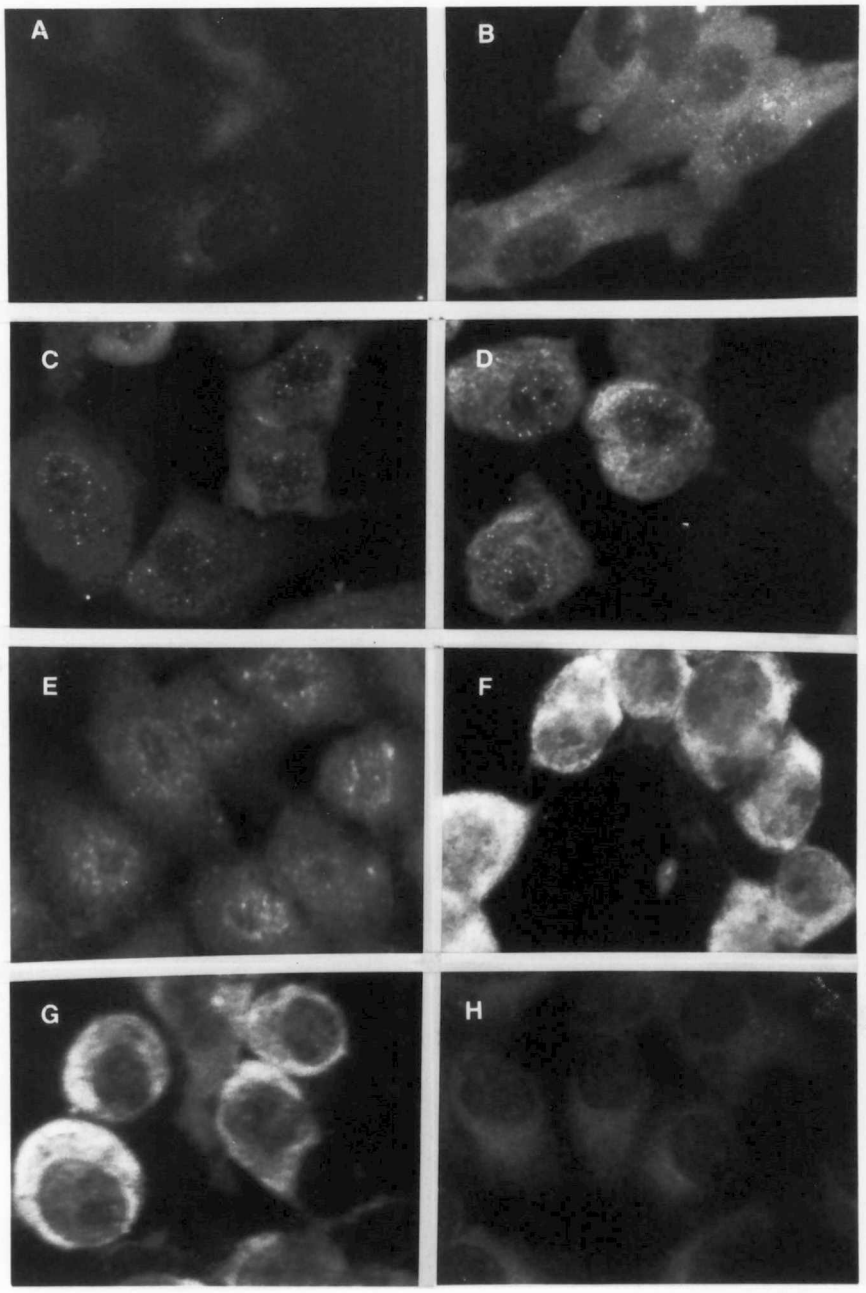

Figure 25.

appearance and migration of ICP0 to the nucleus, but show the nuclear presence of the antigen at later times pi. The antigen is shown (Figure 26) to be maintained in the pre-marginated granular form in the nuclei of cells infected in the presence of azetidine through 10 hrs pi.

HSV-2 infected cells cultured in the presence of canavanine (Figure 27) or azetidine (Figure 28) and stained for immunofluorescence using anti-ICP27 antiserum showed no detectable difference from the antigen distribution in infected cells cultured in the absence of the analogues.

Figure 26. Anti-ICP0 immunofluorescence obtained in HSV-2 infected cells cultured in the presence of azetidine. Vero cells cultured on glass coverslips and incubated in the presence of azetidine were infected with HSV-2 and harvested at various times post infection for immunofluorescent analysis. Panel A: uninfected cells cultured in the presence of azetidine and stained with anti-ICP0 antiserum; Panel B, C, D, and E: HSV-2 infected cells cultured in the presence of azetidine and stained with anti-ICP0 antiserum at 4, 6, 8, and 10 hrs pi, respectively; Panel F: HSV-2 infected cells cultured in the presence of azetidine and stained with pre-immune rabbit serum.

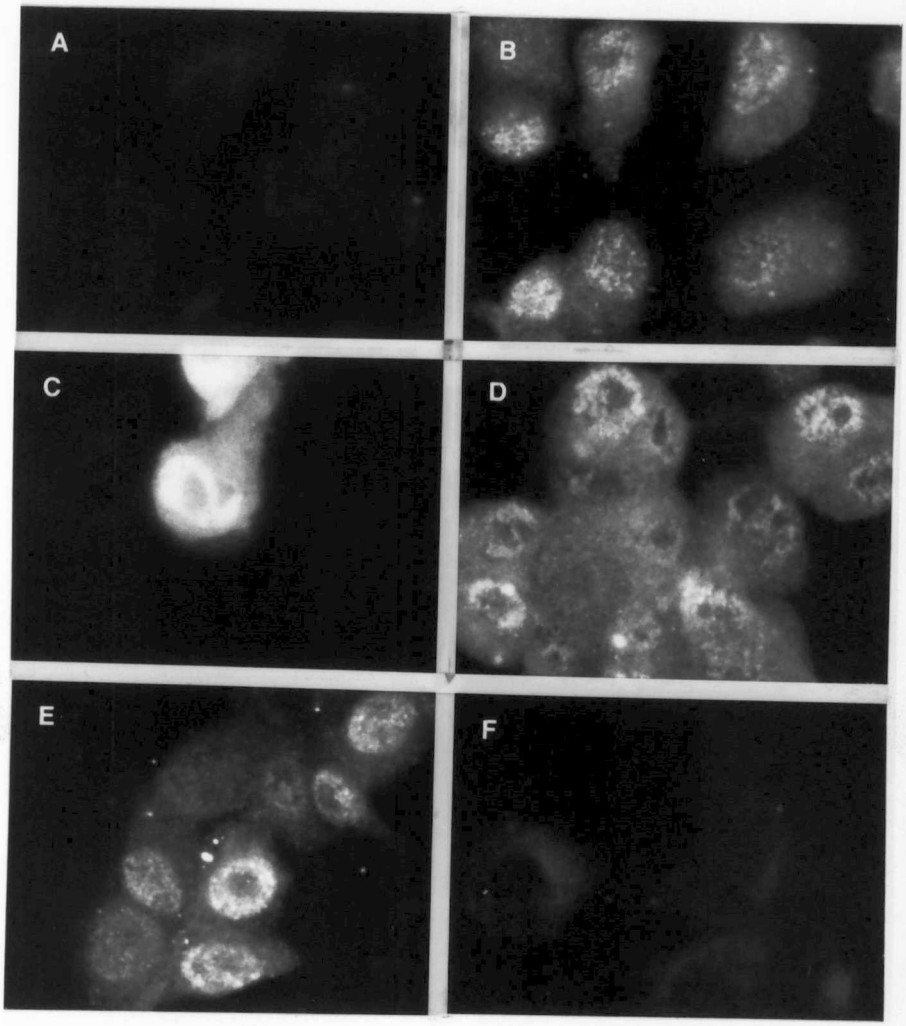

Figure 26.

Figure 27. Anti-ICP27 immunofluorescence obtained in HSV-2 infected cells cultured in the presence of canavanine. Vero cells cultured on glass coverslips and incubated in the presence of canavanine were infected with HSV-2 and harvested at various times post infection for immunofluorescent analysis. Panel A: uninfected cells cultured in the presence of canavanine and stained with anti-ICP27 antiserum; Panel B, C, D, E, and F: HSV-2 infected cells cultured in the presence of canavanine and stained with anti-ICP27 antiserum at 2, 4, 6, 8, and 10 hrs pi, respectively.

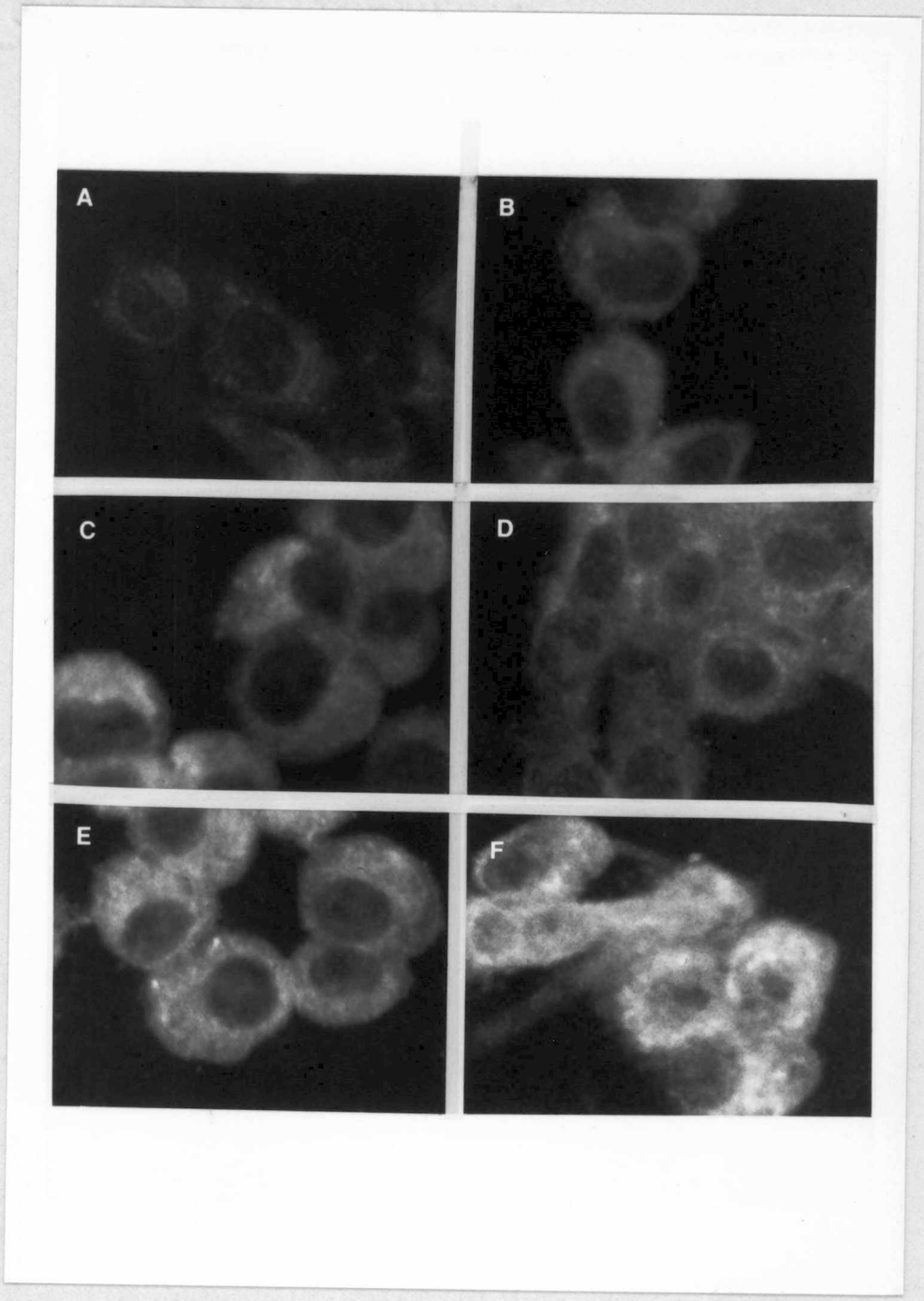

Figure 27.

Figure 28. Anti-ICP27 immunofluorescence obtained in HSV-2 infected cells cultured in the presence of azetidine. Vero cells cultured on glass coverslips and incubated in the presence of azetidine were infected with HSV-2 and harvested at various times post infection for immunofluorescent analysis. Panel A: uninfected cells cultured in the presence of azetidine and stained with anti-ICP27 antiserum; Panel B, C, D, E, and F: HSV-2 infected cells cultured in the presence of azetidine and stained with anti-ICP27 antiserum at 2, 4, 6, 8, and 10 hrs pi, respectively.

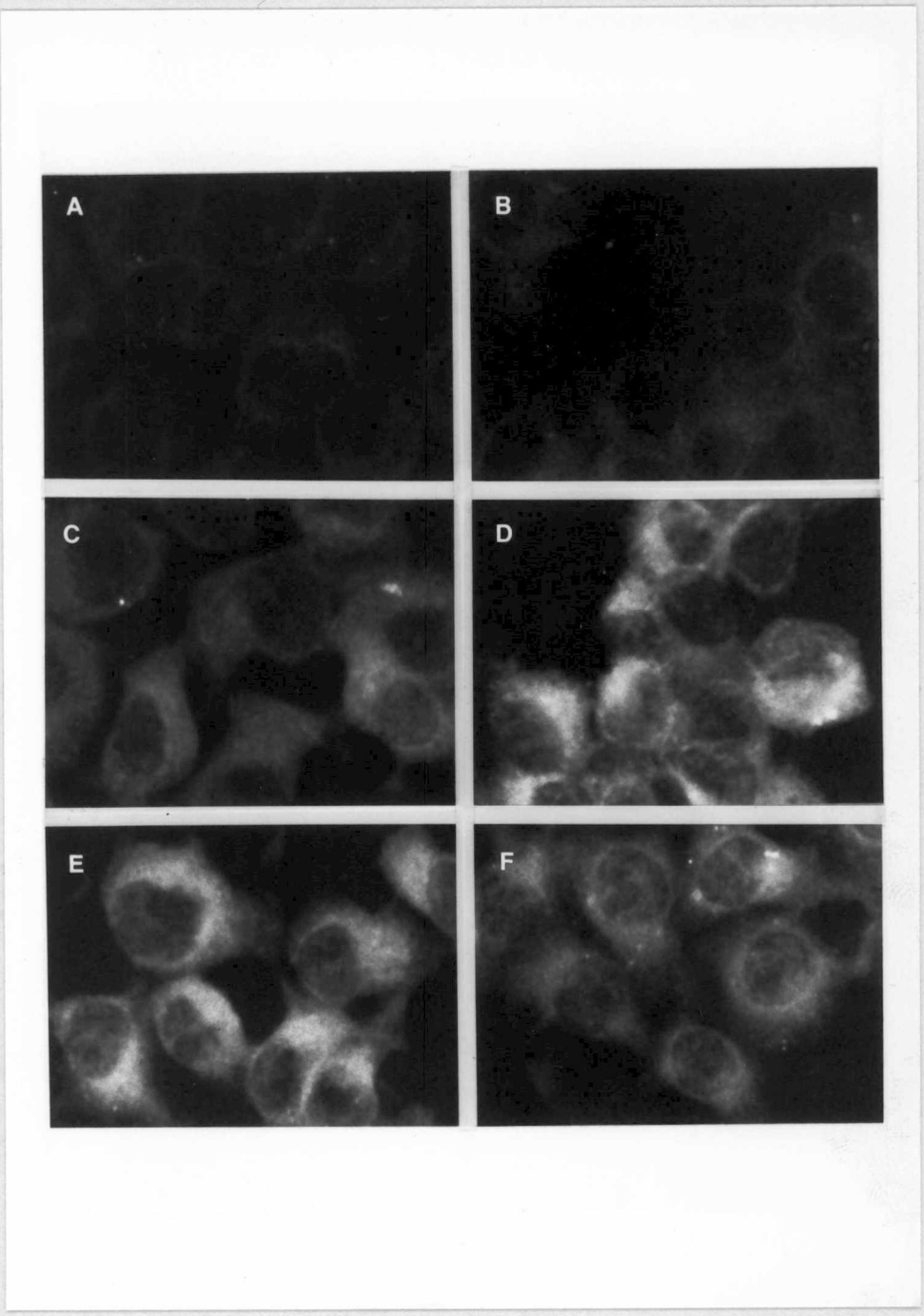

Figure 28.

CHAPTER 4

DISCUSSION

The herpes simplex viruses are large, DNA-containing viruses which are involved with several aspects of medical importance. Virus infections result in the appearance of cutaneous lesions which are self-limiting but very painful. The disappearance of a lesion in a particular location can result in the production of the state of latency, in which the virus is thought to be maintained in a sensory nerve ganglion associated with the original site of infection (Nahmias and Roizman, 1973). Latent infections can, upon the presentation of a proper virus-activating stimulus, result in the recrudescent appearance of the herpes lesion. This recrudescence is usually in the same anatomical site as the original infection and contains infectious virus of the same strain as that involved with the primary infection. The mechanism controlling whether the virus enters either the lytic or latent pathway is not understood. Many laboratories are currently involved in a study of the molecular aspects of gene regulation in eukaryotic systems, and a further understanding of the control systems of the HSV replication scheme will help to further the understanding not only of the HSV system but also of gene regulation in general.

The lytic cycle of the herpes simplex viruses has been the most fruitfully explored of all the HSV-cellular interactions. Although much work has focused on the involvement of the virus with acute herpes virus encephalitis and the possible role of the HSV in the transformation of cells and the induction of cancer, the understanding of these interactions is far from clear. Studies of HSV-specific polypeptide production in cells infected with HSV have resulted in the elucidation of a very well organized replication mechanism consisting of three groups (α, β, and γ) of polypeptides categorized by the kinetics of this synthesis (Honess and Roizman, 1974). These groups of α, β, and γ polypeptides are generally considered to consist of regulatory proteins, proteins enzymatically involved with virus replication, and structural proteins of the virion itself, respectively. Although cells infected with HSV produce up to 44 infected cell polypeptides (Morse, et al., 1978), the number of polypeptides produced in the α class of protein synthesis is very small; and these are all thought to have only regulatory functions (Pereira, et al., 1977). The specific aims of this research project included the production of antisera to each of the purified regulatory α polypeptides synthesized in HSV-2 infected cells and a characterization of these polypeptides as to their intracellular location and times of appearance in the

infected cell. An understanding of the intracellular location of these antigens will help to elucidate the regulatory control mechanisms with which they are involved.

Cells infected with HSV are seen to terminate cellular macromolecular synthesis rapidly and to initiate the production of virus-specific polypeptide synthesis (Sydiskis and Roizman, 1966). The termination of cellular activity is not merely a general "poisoning effect," but is a specific inhibition of certain cellular processes, as is shown by the continued activity of the host RNA polymerase II [used by HSV for the transcription of DNA into mRNA (Costanzo, 1977)] and the use of the cellular glycosylation mechanisms to make viral glycoproteins (E. Wenske, personal communication). Studies aimed at analyzing the cytoplasmic mRNA transcripts made early in the infectious cycle have shown that more than one temporal class of HSV messages are present, even though only the immediate-early polypeptides are translated in large amounts. This finding has provided support for the hypothesis that the regulation of the HSV polypeptide production is under translational control. Findings reported in this thesis tend to support this hypothesis by the discovery that ICP27, a 62,000 m.w. α polypeptide, is isolated exclusively from the cytoplasm of infected cells (Table 1). This finding is different from studies reported by other laboratories (Pereira, et al., 1977)

Table 1. Summary of Results

Characteristic	Polypeptide		
	ICP4	ICP0	ICP27
Maximum time of synthesis	3-6 hrs	2-4 hrs	2-7 hrs
Type specificity	tc	ts	tc
Antigen location:			
No inhibitor	nuc	nuc 1-6 hrs	cyt
Plus canavanine	nuc & cyt	nuc 1-6 hrs	cyt
Plus azetidine	cyt	nuc & cyt	cyt

where a nuclear as well as cytoplasmic involvement of ICP27 was shown by fractionation of cells into nuclear and cytoplasmic components. Work based on cell fractionation alone, however, lacks the specificity obtained with monospecific immunological reagents and is also complicated by leakage of one cell fraction into the other. The indirect immunofluorescent studies presented in this thesis show a marked absence of ICP27 from the nuclei of infected cells. This indicated that the functional involvement of this polypeptide in the regulatory mechanism controlling the synthesis of HSV polypeptides would, of necessity, be associated with a cytoplasmic process. ICP27, a phosphoprotein thought to have kinase activities (Wilcox, 1980), may be involved with the phosphorylation of ribosomal proteins in HSV-infected cells as was shown by Fenwick and Walker (1979). This phosphorylation could be associated with the rapid dissociation of host cell polysomes which was shown to occur soon after infection with HSV (Silverstein and Engelhardt, 1979) and the apparent preferential translation of HSV-specific mRNA.

The possibility that synthesis of the 44 polypeptides of a large virus as complex as HSV is under the control of a single cytoplasmic alteration appears to be a simplistic viewpoint. RNA-DNA hybridization experiments compairing the ratio of hnRNA to polyadenylated cytoplasmic mRNA have shown that specific segments of HSV DNA are

transcribed and transported to the cytoplasm more than other specific DNA segments (Fenwick and Walker, 1978). This information is cited as supportive evidence by the proponents of the theory that HSV polypeptide production is controlled at the level of gene transcription. Polypeptides involved with a transcriptional control mechanism in HSV infected cells should express a nuclear location as the site of their functional importance. Several laboratories have shown that α polypeptides have to be produced in functionally active form (Honess and Roizman, 1975; Pereira, et al., 1977) and transported to the nucleus (Courtney and Benyesh-Melnick, 1974) in order to initiate the synthesis of β polypeptides and the subsequent events of HSV polypeptide synthesis. The finding that amino acid analogues, used to produce non-functional α polypeptides, inhibited the transition from α polypeptide production to β polypeptide production supported the hypothesis that one or more α polypeptides were functionally involved in controlling the cascade regulation of HSV polypeptide synthesis. Courtney and Benyesh-Melnick (1974) used a conditional lethal mutant of HSV-1 (tsB2) with a ts lesion in the gene coding for ICP4 to show that processing of the polypeptide to its highest molecular weight form and the concurrent migration of the polypeptide to the nucleus were necessary for the initiation

of synthesis of the next group of polypeptides. Results reported in this study agree very well with the amino acid analogue and tsB2 results reported above. An analogue of proline and hydroxyproline (azetidine) was shown by PAGE to inhibit the processing of ICP4 from the lower molecular weight component to its higher molecular weight form (Figure 8, page 67). This amino acid analogue was thus shown to produce the same apparent effects on the processing of ICP4 as did the ts lesion found in tsB2. The alterations produced in the cascade regulation of HSV polypeptide synthesis in cells cultured in the presence of azetidine were also similar to those shown for tsB2 infected cells cultured at the non-permissive temperature. Immunofluorescent analysis of ICP4 produced in cells infected with HSV-2 and cultured in the presence of azetidine clearly show that the amino acid analogue inhibits the normal migration of ICP4 to the nuclei of infected cells. Cytoplasmic fluorescence seen in these infected cells indicates the continued translation of messenger RNA transcripts specifying the lower molecular weight form of ICP4. Thus, ICP4, an immediate-early polypeptide produced in HSV infected cells and thought to be involved with the regulation of HSV protein synthesis, probably performs its function at the transcriptional level in HSV infected cell nuclei.

The α class of HSV-2 polypeptides, comprised of only three polypeptides (ICP4, ICP0, and ICP27), are the first virus-specific polypeptides produced in HSV-2 infected cells. These polypeptides are transcribed by the host cell RNA polymerase II (Costanzo, 1977), as, presumably, are the other HSV polypeptides. One possible hypothesis for the mechanism controlling HSV protein synthesis is that the functional higher molecular weight form of ICP4 is needed to alter the site specificity of the RNA polymerase II molecule so that it recognizes β and γ polypeptide gene promoters. In the absence of the functional nuclear form of ICP4, the transcription of α polypeptide messages would continue and be translated at normal rates, resulting in the accumulation of α polypeptides seen in cells infected with the HSV-1 tsB2 mutant or HSV infected cells cultured in the presence of various metabolic inhibitors. The processing of ICP4 to its higher molecular weight form may be associated with the autokinase activities suggested for ICP4 by Wilcox, et al. (1980). If this were the case, no other HSV or cellular polypeptide would be needed to assist in the modification of the polypeptide.

An alternative explanation for the function of the higher molecular weight form of ICP4 is that it binds to the HSV DNA in such a manner as to make the promotors of the β and γ polypeptide classes more readily available

to the RNA polymerase II, and would thus increase the rate of transcription of these genes. This hypothesis could be supported by the work of Powell and Purifoy (1976) which has demonstrated the DNA binding properties of the polypeptide. The results of Cabral, et al. (1980) suggest that the ICP4 polypeptide is associated at the ultrastructural level with the infected cell chromatin. This association results in the margination of the polypeptide to the periphery of infected nuclei by 8 to 10 hrs pi as has been shown by both immunoelectronmicroscopy and immunofluorescence.

The last remaining member of the α polypeptide class was also the last HSV infected cell polypeptide to be recognized as a distinct HSV-specific protein. The detection of this polypeptide was obscured by the comigration of a polypetide (ICP8) which is a member of the β polypeptide class of infected cell polypeptides. ICP0 is made in small amounts and is only detectable through the first 6 hrs pi (Table 1, page 127) at which time it is rapidly degraded. Results obtained in this study support the very short time of appearance of the polypeptide and have placed it in the nuclei of the infected cells (Figure 17, page 95). The polypeptide has been shown by immunofluorescence to marginate and decrease in fluorescent intensity at 5 and 6 hrs pi.

Attempts to assign a functional importance to ICP0 have met with little success. Conditional lethal mutants having a mutation in the gene coding for ICP0 have never been isolated, despite attempts by numerous laboratories to isolate immediate-early polypeptide mutants. This difficulty could be due to the ICP0 gene being comprised of an unusually stable sequence of nucleotides, one into which it is very difficult to induce mutations. Another explanation could be that the ICP0 polypeptide is actually a protein which has no significance at all to the lytic cycle of the HSV infection and thus would not be selected for under conditions used to isolate conditional lethal mutants. A comparison of results obtained from HSV infected cells cultured in the presence of amino acid analogues with those obtained from tsB2 infected cells cultured at the non-permissive temperature support the lack of involvement of ICP0 in the HSV lytic cycle. ICP0 produced in the presence of amino acid analogues should be non-functional, whereas the ICP0 produced in cells infected with tsB2 and cultured at the non-permissive temperature should have normal functional capabilities because the ts lesion in tsB2 is known to involve only the gene coding for ICP4. The effects of altering infected cell polypeptide synthesis in either of these two ways are indifferentiable; thus any lytic cycle function performed by the normal ICP0 polypeptide produced

in tsB2 infected cells is not detectable by these assays, or simply does not exist. The functional importance of this polypeptide has not been ascertained in this study, but a hypothesis linking the nuclear involvement of ICP4 and ICP0 with control of the HSV lytic and latent states of infection will be proposed.

Cascade regulation of HSV infected cell polypeptide production involves the co-ordinated control of three kinetic classes of HSV-specific polypeptides. The α polypeptides are functionally important in the regulation of HSV protein synthesis. By placing two of the α polypeptides (ICP4 and ICP0) in the nuclei of HSV-2 infected cells and the third α polypeptide (ICP27) in the cytoplasm of HSV-2 infected cells, this study has produced major candidates for mediators of both the transcriptional and translational levels of control of polypeptide synthesis. Because of the inverse relationship of the time of appearance of ICP0 and ICP4 in the nuclei of infected cells entering the lytic cell (Table 1, page 127) it is interesting to consider the possibility that ICP0 is involved in some aspect of a virus infection other than the lytic cycle. The control system sending the cell either into the lytic or latent states should logically operate at the immediate-early polypeptide stage. This would be the most efficient time to initiate latency as it would alleviate the unnecessary production

of virus structural components and would also lessen the deleterious effects a lytic virus infection would have on the host cell. The control signal initiating the latent state is not known but could involve an alteration of the early events in cascade regulation of HSV-specific polypeptides. ICP0, an α polypeptide for which no lytic function has been ascribed, is a very likely candidate for the molecular signal directing the induction of the latent state. The intracellular conditions determining whether ICP4 would predominate and produce a lytic infection or ICP0 would predominate and produce a latent infection are not known but may be involved with a cellular factor because of the apparent relationship between sensory ganglion nerve cells and the latent state or epithelial cells and the lytic cycle. Experiments designed to determine the possible involvement of ICP0 with a control mechanism leading to the latent state could be performed using deletion mutants in which the ICP0 gene has been partially or totally removed. An analysis of the ability of this virus to produce latency in in vivo animal systems would be instructive as to the involvement of ICP0 with the latent state.

LIST OF REFERENCES

LIST OF REFERENCES

Abodeely, R. A., L. A. Lawson, and C. C. Randall. 1970. Morphology and entry of enveloped and deenveloped equine abortion (herpes) virus. J. Virol. 5: 513-523.

Anderson, K. P., J. R. Stringer, L. E. Holland, and E. K. Wagner. 1979. Isolation and localization of herpes simplex virus type 1 mRNA. J. Virol. 30: 805-820.

Andrewes, C. H., H. G. Pereira, and P. Wildy. 1978. Viruses of Vertebrates. 4th Edition. London: Bailliere Tindall.

Aurelian, L. 1973. Virions and antigens of herpesvirus type 2 in cervical carcinoma. Cancer Res. 33: 1539.

Aurelian, L., J. D. Strandbe, L. V. Melendez, and L. V. Johnson. 1971. Herpesvirus type 2 isolated from cervical tumor cells grown in tissue culture. Science 174: 704.

Ben-Porat, T., J. Jong-ho, and A. S. Kaplan. 1974. Early functions of the genome of herpesvirus. IV. Fate of translation of immediate early viral mRNA. Virol. 59: 524-531.

Benzeev, A., and Y. Becker. 1975. Symmetrical transcription of herpes-simplex virus DNA in infected BSC-1 cells. Nature 254: 719-722.

Bookout, J., I. Hirsch, D. J. M. Purifoy, and N. Biswal. 1979. Herpes simplex virus types 1 and 2: Comparison of the defective genomes and virus-specific polypeptides. Virol. 93: 598-604.

Bookout, J. B., and C. C. Levy. 1980. Comparative examination of the polypeptides of herpes simplex virus: types 1 and 2. Virol. 101: 198-216.

Camacho, A. and P. G. Spear. 1978. Transformation of hamster embryo fibroblasts by a specific fragment of the herpes simplex virus genome. Cell 15: 993.

Cassai, E. N., M. Sarmiento, and P. G. Spear. 1975. Comparison of virion proteins specified by herpes simplex virus types 1 and 2. J Virol. 16: 1327-1331.

Chartrand, P., N. D. Stow, M. C. Timbury, and N. M. Wilkie. 1979. Physical mapping of *paa*^{r} mutations of herpes simplex virus type 1 and type 2 by intertypic marker rescue. J. Virol. 31: 265-276.

Collard, W., H. Thornton, and M. Green. 1973. Cells transformed by human herpesvirus type 2 transcribed virus- specific RNA sequences shared by herpesvirus types 1 and 2. Nature, Biol. 243: 264.

Costanzo, T., G. Campadelli-Fiume, L. Foa-Tomase, and E. Cassai. 1977. Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. J. Virol. 21: 996-1001.

Courtney, R. J. 1976. Immunological and biochemical characterization of individual polypeptides induced by herpes simplex virus types 1 and 2. In Cancer Biology. III. Herpes Viruses Epidemiology, Molecular Events, Oncogenicity and Therapy. Edited by C. Borek and D. W. King. New York: Stratton Intercontinental Medical Corp., Advances in Pathobiology 5: 87-103.

Courtney, R. J., and M. Benyesh-Melnick. 1974. Isolation and characterization of a large molecular weight polypeptide of herpes simplex virus type 1. Virol. 62: 539-551.

Courtney, R. J., R. M. McCombs, and M. Benyesh-Melnick. 1970. Antigens specified by herpesviruses. 1. Effect of arginine deprivation on antigen synthesis. Virol. 40: 379-386.

Courtney, R. J., R. M. McCombs, and M. Benyesh-Melnick. 1971. Antigens specified by herpesviruses. 2. Effect of arginine deprivation on synthesis of cytoplasmic and nuclear proteins. Virol. 43: 256-265.

Courtney, R. J., and K. L. Powell. 1975. Immunological and biochemical characterization of polypeptides induced by herpes simplex virus types 1 and 2. In Oncogenesis and Herpesviruses II, pp. 63-73. Edited by G. de-The, M. A. Epstein, and H. zurHausen. Lyon, France: International Agency for Research on Cancer.

Courtney, R. J., P. A. Schaffer, and K. L. Powell. 1976. Synthesis of virus-specific polypeptides by temperature-sensitive mutants of herpes simplex virus type 1. Virol. 75: 306-318

Dales, S., and H. Silverberg. 1969. Viropexis of herpes simplex virus by HeLa cells. Virol. 37: 475-480.

Davidson, R. L., S. J. Adelstein, and M. N. Oxman. 1973. Herpes simplex virus as a source of thymidine kinase-deficient mouse cells: suppression and reactivation of the viral enzyme. Proc. Natl. Acad. Sci. (USA) 70: 1912.

Davis, B. J. 1964. Disc electrophoresis. II. Method and application to serum proteins. Ann. N. Y. Acad. Sci. 121: 404-427.

Dimmock, N. J., and D. H. Watson. 1969. Proteins specified by influenza virus in infected cells: analysis by polyacrylamide gel electrophoresis of antigens not present in the virus particle. J. Gen. Virol. 5: 499-509.

Dreesman, G. R., and M. Benyesh-Melnick. 1967. Spectrum of human cytomegalovirus complement-fixing antibodies. J. Immunol. 99: 1106-1114.

Duff, R., and Rapp, F. 1971. Oncogenic transformation of hamster cells after exposure to herpes simplex virus type 2. Nature New Biol. 233: 48.

Duff, R., and Rapp, F. 1973. Oncogenic transformation of hamster embryo cells after exposure to inactivated herpes simplex virus type 1. J. Virol. 12: 209.

Easton, A. J., and J. B. Clements. 1980. Temporal regulation of herpes simplex virus type 2 transcription and characterization of virus immediate-early mRNA's. Nucleic Acids Research 8: 2627-2645.

Eberle, R., and R. J. Courtney. 1981. Assay of type-specific and type-common antibodies to herpes simplex virus types 1 and 2 in human sera. Infect. and Immun. 31: 1062-1070.

Esparza, J., M. Benyesh-Melnick, and P. A. Schaffer. 1976. Intertypic complementation and recombination between temperature-sensitive mutants of herpes simplex virus type 1 and type 2. Virol. 70: 372-384.

Feighny, R. J., M. P. Farrell, and J. S. Pagano. 1980. Polypeptide synthesis and phosphorylation in Epstein-Barr virus-infected cells. J. Virol. 34: 455-463.

Fenwick, M., L. S. Morse, and B. Roizman. 1979. Anatomy of herpes simplex virus DNA. XI. Apparent clustering of functions effecting rapid inhibition of host DNA and protein synthesis. J. Virol. 29: 825-827.

Fenwick, M., and B. Roizman. 1977. Regulation of herpesvirus macromolecular-synthesis. 6. Synthesis and modification of viral polypeptides in enucleated cells. J. Virol. 22: 720-725.

Fenwick, M. L., and M. J. Walker. 1978. Suppression of the synthesis of cellular macromolecules by herpes simplex virus. J. Gen. Virol. 41: 37-51.

Fenwick, M. L., and M. J. Walker. 1979. Phosphorylation of a ribosomal protein and of virus-specific proteins in cells infected with herpes simplex virus. J. Gen. Virol. 45: 397-405

Fenwick, M., M. Walker, and L. Marshall. 1980. Some characteristics of an early protein (ICP22) synthesized in cells infected with herpes simplex virus. J. Gen. Virol. 47: 333-341.

Fenwick, M. L., M. J. Walker, and J. M. Petkevich. 1978. On the association of virus proteins with the nuclei of cells infected with herpes simplex virus. J. Gen. Virol. 39: 519-529.

Flannery, V. L., R. J. Courtney, and P. A. Schaffer. 1977. Expression of an early, nonstructural antigen of herpes simplex virus in cells transformed in vitro by herpes simplex virus. J. Virol. 21: 284-291.

Frenkel, N., and B. Roizman. 1972. Separation of herpesvirus deoxyribonucleic acid duplex into unique fragments and intact strand on sedimentation in alkaline gradients. J. Virol. 10: 565-572.

Frenkel, N., R. J. Jacob, R. W. Honess, G. S. Hayward, H. Locker, and B. Roizman. 1975. Anatomy of herpes simplex virus DNA. 3. Characterization of defective DNA molecules and biological properties of virus populations containing them. J. Virol. 16: 153-167.

Frenkel, N., H. Locker, B. Cox, B. Roizman, and F. Rapp. 1976. Herpes simplex virus DNA in transformed cells: sequence complexity in five hamster cell lines and one derived hamster tumor. J. Virol. 18: 885-893.

Frenkel, N., H. Locker, W. Batterson, G. S. Hayward, and B. Roizman. 1976. Anatomy of herpes simplex virus DNA. 6. Defective DNA originates from S component. J. Virol. 20: 527-531.

Frenkel, N., and B. Roizman. 1972. Ribonucleic acid synthesis in cells infected with herpes simplex virus. 5. Controls of transcription and of RNA abundance. Proc. Natl. Acad. Sci. (USA) 69: 2654-2658.

Furlong, D., H. Swift, and B. Roizman. 1972. Arrangement of herpesvirus deoxyribonucleic acid in the core. J. Virol. 10: 1071-1074.

Gibson, W., and B. Roizman. 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. 10: 1044-1052

Grafstrom, R. H., J. C. Alwine, W. L. Steinhart, C. W. Hill, and R. W. Hyman. 1975. The terminal repetition of herpes simplex virus DNA. Virol. 67:144-157.

Halliburton, I. W. 1980. Intertypic recombinants of herpes simplex viruses. J. Gen. Virol. 48: 1-23.

Halliburton, I. W., R. E. Randall, R. A. Killington, and D. H. Watson. 1977. Some properties of recombinants between type 1 and type2 herpes simplex viruses. J. Gen. Virol. 36: 471-484.

Halliburton, I. W., and M. C. Timbury. 1976. Temperature-sensitive mutants of herpes simplex virus type 2. Description of 3 new complementation groups and studies on inhibition of host cell DNA synthesis. J. Gen. Virol. 30: 145-148.

Hamada, C., and A. S. Kaplan. 1965. Kinetic synthesis of various types of antigen proteins in cells infected with pseudorabies virus. J. Bacteriol. 89: 1328.

Hay, R. T., and J. Hay. 1980. Properties of herpesvirus-induced "immediate early" polypeptides. Virol. 104: 230-234.

Holland, L. E., K. P. Anderson, J. R. Stringer, and E. K. Wagner. 1979. Isolation and localization of herpes simplex virus type 1 mRNA abundant before viral DNA synthesis. J. Virol. 31: 447-462.

Hollinshead, A. C., O. Lee, P. B. Chretien, J. L. Tarpley, W. E. Rawls, and E. Adam. 1973. Antibodies to herpesvirus nonvirion antigens in squamous carcinomas. Science 182: 713.

Honess, R. W., and B. Roizman. 1973. Proteins specified by herpes simplex virus. II. Identification and relative molar rates of synthesis of structural and non-structural herpesvirus polypepitdes in infected cells. J. Virol. 12: 1347-1365.

Honess, R. W., and B. Roizman. 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. 14: 8-19.

Honess, R. W., and B. Roizman. 1975. Regulation of herpesvirus macromolecular synthesis. 3. Sequential transition of polypeptide synthesis requires functional viral polypeptides. J. Virol. 16: 1308-1326.

Honess, R. W., and D. H. Watson. 1977. Unity and diversity in herpesviruses. J. Gen. Virol. 37: 15-37.

Hummeler, K., N. Tomassini, and B. Zajac. 1969. Early events in herpes simplex virus infection: a radiographic study. J. Virol. 4: 67-74.

Jacquemont, B., A Garcia, and J. Huppert. 1980. Nuclear processing of viral high molecular weight RNA in cells infected with herpes simplex virus type 1. J. Virol. 35: 382-389.

Jacquemont, B., and B. Roizman. 1975. Ribonucleic acid synthesis in cells infected with herpes simplex virus. Characterization of viral high molecular weight nuclear RNA. J. Gen. Virol. 29: 155-165.

Jones, P. C., and B. Roizman. 1979. Regulation of herpesvirus macromolecular synthesis: VIII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. J. Virol. 31: 299-314.

Kaplan, A. S., H. Shimono, and T. Ben-Porat. 1970. Synthesis of proteins in cells infected with herpesvirus. III. Relative amino acid content of various proteins formed after infection. Virol. 40: 90-101.

Kessler, I. I. 1974. Perspectives on the epidemiology of cervical cancer with special reference to the herpesvirus hypothesis. Cancer Res. 34: 1091-1110.

Kieff, E., B. Hoyer, S. Bachenhetti, and B. Roizman. 1972. Genetic relatedness of type 1 and type 2 herpes simplex viruses. J. Virol. 9: 738-745.

Killington, R. A., J. Yeo, R. W. Honess, D. H. Watson, B. E. Duncan, I. W. Halliburton, adn J. Munford. 1977. Comparative analysis of proteins and antigens of 5 herpesviruses. J. Gen. Virol. 37: 297-310.

Kimura, S., V. L. Flannery, B. Levy, and P. A. Schaffer. 1975. Oncogenic transformation of primary hamster cells by herpes simplex virus type 2 (HSV-2) and an HSV-2 temperature-sensitive mutant. Int. J. Cancer 15: 786.

Klein, R. J. 1976. Pathogenic mechanisms of recurrent herpes simplex virus infections. Brief review. Arch. Virol. 51: 1-13.

Knipe, D. M., W. T. Ruyechan, B. Roizman, and I. W. Halliburton. 1978. Molecular genetics of herpes simplex virus: demonstration of regions of obligatory and non-obligatory identity within diploid regions of the genome by sequence replacement and insertion. Proc. Natl. Acad. Sci. (USA) 75: 3896-3900.

Kozak, M., and B. Roizman. 1974. Regulation of herpesvirus macromolecular synthesis: nuclear retention of nontranslated viral RNA sequences. Proc. Natl. Acad. Sci. (USA) 71: 4322-4326.

Kozak, M., and B. Roizman. 1975. RNA-synthesis in cells infected with herpes simplex virus. 9. Evidence for accumulation of abundant symmetric transcripts in nuclei. J. Virol. 15: 36-40.

Kucera, L. S., and J. P. Gudson. 1976. Transformation of human embryonic fibroblast cells with dye-light inactivated herpesvirus type 2. Gynecol. Inv. 6: 42.

Kucera, L. S., and J. P. Gudson. 1976. Transformation of human embyronic fibroblasts by photodynamically inactivated herpes simplex virus type 2 at supra-optimal temperature. J. Gen. Virol. 30: 257.

Kucera, L. S., J. P. Gudson, I. Edwards, and G. Herbst. 1977. Oncogenic transformation of rat embryo fibroblasts with photoinactivated herpes simplex virus: rapid in vitro cloning of transformed cells. J. Gen. Virol. 35: 473-485.

Leiden, J. M., N. Frenkel, and F. Rapp. 1980. Identification of the herpes simplex virus DNA sequences present in six herpes simplex virus thymidine kinase-transformed mouse cell lines. J. Virol. 33: 272-285.

Leung, W. C. 1980. Cooperation between herpes simplex virus specific α protein and host cell RNA polymerase II in the transcription of viral deoxypyrimidine kinase. Can. J. Microbiol. 26: 401-404.

Lewis, J. G., L. Kucera, R. Eberle, and R. J. Courtney. 1981. Detection of herpes simplex virus type 2 glycoproteins expressed in virus-transformed rat cells. Submitted for publication.

Li, J. H., M. A. Jerkofsky, and F. Rapp. 1975. Demonstration of oncogenic potential of mammalian cells transformed by DNA-containing viruses following photodynamic inactivation. Int. J. Cancer 15: 190.

Ludwig, H. O., N. Biswal, and M. Benyesh-Melnick. 1972. Studies on relatedness of herpesviruses through DNA-DNA hybridization. Virol. 49: 95-101.

Macnab, J. C. M. 1974. Transformation of rat embryo cells by temperature-sensitive mutants of herpes simplex virus. J. Gen. Virol. 24: 143.

Matis, J., and J. Rajcani. 1980. Preparation of immune serum to immediate early and early polypeptides specified by herpes simplex virus type 1. Acta. Virol. 24: 105-113.

Melnick, J. L., R. J. Courtney, K. L. Powell, P. A. Schaffer, M. Benyesh-Melnick, G. R. Dreesman, T. Anzai, and E. Adam. 1976. Studies on herpes simplex virus and cancer. Cancer Res. 36: 845-856.

Minson, A. C., G. K. Darby, and P. Wildy. 1979. Virus specific DNA sequences present in cells which carry the herpes simplex virus thymidine kinase gene. J. Gen. Virol. 45: 489.

Minson, A. C., M. E. Thoules, R. P. Eglin, and G. Darby. 1976. The detection of virus DNA sequences in a herpes type 2 transformed hamster cell line. Int. J. Cancer 17: 493-500.

Morgan, C., S. A. Ellison, H. M. Rose, and D. H. Moore. 1954. J. Exp. Med. 100: 195.

Morgan, C., H. M. Rose, and B. Mednis. 1968. Electron microscopy of herpes simplex virus. I. Entry. J. Virol. 2: 507.

Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer. 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 X HSV-2 recombinants. J. Virol. 26: 389-410.

Munyon, W., E. Kraiselburd, D. Davis, and J. Mann. 1971. Transformation of thymidine kinaseless L cells by infection with ultraviolet-irradiated herpes simplex virus. J. Virol. 7: 813.

Nahmias, A. J., and B. Roizman. 1973. Infection with herpes simplex viruses-1 and viruses-2. 1. N. Eng. J. Med. 389: 667.

Naib, Z. M., A. J. Nahmias, and J. E. Josey. 1966. Cytology and histopathology of cervical herpes simplex infection. Cancer 19: 1026.

Notter, M. F. D., and J. J. Docherty. 1976. Reaction of antigens isolated from herpes simplex virus-transformed cells with sera of squamous-cell carcinoma patients. Cancer Res. 36: 4394-4401.

Ojeda, V. J. 1980. Fatal herpes simplex encephalitis with demonstration of virus in the olfactory pathway. Pathology 12: 429-437.

Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. Ann. N. Y. Acad. Sci. 121: 321-349.

Pereira, L., E. Cassai, R. W. Honess, B. Roizman, M. Terni, and A. Nahmias. 1976. Variability in the structural polypeptides of herpes simplex virus 1 strains: potential application in molecular epidemiology. Infection and Immunity 13: 211-220.

Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman. 1977. Regulation of herpesvirus macromolecular synthesis. 5. Properties of alpha polypeptides made in HSV-1 and HSV-2 infected cells. Virol. 77: 733-749.

Pignatti, P. F., E. Cassai, and U. Bertazzoni. 1979. Herpes simplex virus DNA synthesis in a partially purified soluble extract from infected cells. J. Virol. 32: 1033-1036.

Porter, D. D., I. Wimberly, and M. Benyesh-Melnick. 1969. Prevalence of antibodies to EB virus and other herpesviruses. J. Am. Med. Assoc. 208: 1675.

Powell, K. L., A. Buchan, C. Sim, and D. H. Watson. 1974. Type-specific protein in herpes simplex virus envelope reacts with neutralizing antibody. Nature (London) 249: 360-361.

Powell, K. L., and R. J. Courtney. 1975. Polypeptides synthesized in herpes simplex virus type 2 infected HEp-2 cells. Virol. 66: 217-228.

Powell, K. L., R. Mirkovic, and R. J. Courtney. 1977. Comparative analysis of polypeptides induced by type 1 and type 2 strains of herpes simplex virus. Intervirol. 8: 18-29.

Powell, K. L., and D. J. M. Purifoy. 1976. DNA-binding proteins of cells infected by herpes simplex virus type 1 and type 2. Intervirol. 7: 225-239.

Powell, K. L., and D. J. M. Purifoy. 1977. Non-structural proteins of herpes simplex virus. 1. Purification of induced DNA-polymerase. J. Virol. 24: 618-626.

Powell, K. L., D. J. M. Purifoy, and R. J. Courtney. 1975. The synthesis of herpes simplex virus proteins in the absence of virus DNA synthesis. Bioc. Biop. R. 66: 262-271.

Preston, C. M. 1979a. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild type virus or the temperature-sensitive mutant tsK. J. Virol. 29: 275-284.

Preston, C. M. 1979b. Abnormal properties of an immediate early polypeptide in cells infected with the herpes simplex virus type 1 mutant tsK. J. Virol. 32: 357-369.

Preston, V. G., A. J. Davidson, H. S. Marsden, M. C. Timbury, J. H. Subak-Sharpe, and N. M. Wilkie. 1978. Recombinants between herpes simplex virus types 1 and 2: analysis of genome structures and expression of immediate early polypeptides. J. Virol. 28: 499-517.

Purifoy, D. J. M., and K. L. Powell. 1976. DNA-binding proteins induced by herpes simplex virus type 2 in HEp-2 cells. J. Virol. 19: 717-731.

Purifoy, D. J. M., and K. L. Powell. 1977. Herpes simplex virus-DNA polymerase as site of phosphonoacetate sensitivity - temperature-sensitive mutants. J. Virol. 24: 470-477.

Rawls, W. E., K. Iwamoto, E. Adam, and J. L. Melnick. 1970. Measurement of antibodies to herpesvirus types 1 and 2 in human sera. J. Immunol. 104: 599-606.

Rawls, W. E., W. A. Tompkins, M. E. Figueroa, and J. L. Melnick. 1968. Herpesvirus type 2. Association with carcinoma of the cervix. Science 161: 1255-1256.

Roizman, B. 1979. The structure and isomerization of herpes simplex virus genomes. Cell 16: 481-494.

Roizman, B., and E. D. Kieff. 1975. Herpes simplex and Epstein-Barr viruses in human cells and tissues: a study in contrasts. In Cancer: A Comprehensive Treatise, Vol. 2, p. 241. Edited by F. F. Becker. New York: Plenum Press.

Ruyechan, W. T., L. S. Morse, D. M. Knipe, and B. Roizman. 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. 29: 677-697.

Schaffer, P. A., G. M. Aron, N. Biswal, and M. Benyesh-Melnick. 1973. Temperature-sensitive mutants of herpes simplex virus type 1. Isolation, complementation, and partial characterization. Virol. 52: 57-71.

Schaffer, P. A., D. R. Bone, and R. J. Courtney. 1976. DNA-negative temperature sensitive mutants of herpes simplex virus type 1. Patterns of viral DNA synthesis after temperature shift-up. J. Virol. 17: 1043-1048.

Silverstein, S., and D. L. Engelhardt. 1979. Alterations in the protein synthetic apparatus of cells infected with herpes simplex virus. Virol. 95: 334-342.

Sim, C., and D. H. Watson. 1973. Role of type specific and cross reacting structural antigens in neutralization of herpes simplex virus types 1 and 2. J. Gen. Virol. 19: 217-233.

Skare, J., W. P. Summers, and W. C. Summers. 1975. Structure and function of herpes virus genomes. 1. Comparison of 5 HSV-1 and 2 HSV-2 strains by cleavage of their DNA with Eco-R1 restriction endonuclease. J. Virol. 15: 814-816.

Smith, K. O. 1964. Relationships between the envelope and infectivity of herpes simplex viruses. Proc. Soc. Exp. Biol. Med. 115: 814-816.

Spear, P. G., and B. Roizman. 1968. The proteins specified by herpes simplex virus. 1. Time of synthesis, transfer into nuclei, and properties of proteins made in productively infected cells. Virol. 36: 545-555.

Spear, P. G., and B. Roizman. 1972. Proteins specified by herpes simplex viruses. V. Purification and structural proteins of the herpes virion. J. Virol. 9: 431.

Spear, P. G., and B. Roizman. 1980. Herpes simplex viruses. In Molecular Biology of Tumor Viruses, 2nd Edition, Part 2: DNA Tumor Viruses, pp. 615-746. Edited by J. Tooze. New York: Cold Spring Harbor Laboratory.

Strnad, B. C., and L. Aurelian. 1978. Proteins of herpesvirus type 2. III. Isolation and immunologic characterization of a large molecular weight viral protein. Virol. 87: 401-415.

Subak-Sharpe, H., and J. Hay. 1965. An animal virus with DNA of high arginine and cytosine content which codes for S RNA. J. Mol. Biol. 12: 924.

Sugino, W. M., and D. T. Kingsbury. 1976. DNA homologies between strains of herpes simplex virus. Virol. 71: 605-608.

Summers, W. C. 1980. Molecular epidemiology of DNA viruses: applications of restriction endonuclease cleavage site analysis. Yale J. Biol. and Med. 53: 55-59.

Swanstrom, R. I., K. Pivo, and E. K. Wagner. 1975. Restricted transcription of herpes simplex virus genome occuring early after infection and in presence of metabolic inhibitors. Virol. 66: 140-150.

Sydiskis, R. J., and B. Roizman. 1967. The disaggregation of host polyribosomes in productive and abortive infection with herpes simplex virus. Virol. 32: 678-686.

Sydiskis, R. J., and B. Roizman. 1968. The sedimentation profiles of cytoplasmic polyribosomes in mammalian cells productively and abortively infected with herpes simplex virus. Virol. 34: 562-565.

Talley-Brown, S., and R. L. Millette. 1979. Gene expression of herpes simplex virus. 1. Analysis of cytoplasmic RNA's in infected Xeroderma Pigmentosum cells. J. Virol. 31: 733-740.

Timbury, M. C., and J. H. Subak-Sharpe. 1973. Genetic interactions between temperature sensitive mutants of types 1 and 2 herpes simplex viruses. J. Gen. Virol. 18: 347-357.

Wagner, E. K., and B. Roizman. Ribonucleic acid synthesis in cells infected with herpes simplex virus. J. Virol. 4: 36-46.

Wagner, E. K., R. I. Swanstrom, and M. G. Stafford. 1972. Transcription of herpes simplex virus genome in human cells. J. Virol. 10: 675.

Watson, R. J., C. M. Preston, and J. B. Clements. 1979. Separation and characterization of herpes simplex virus type 1 immediate-early mRNA's. J. Virol. 31: 42-52.

Watson, D. H., W. C. Russell, and P. Wildy. 1963. Virol. 19: 250.

Wilcox, K. W., A Kohn, E. Sklyanskaya, and B. Roizman. 1980. Herpes simplex virus phosphoproteins. 1. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. 33: 167-182.

APPENDIX

PREPARATION OF SODIUM DODECYL SULFATE (SDS)-POLYACRYLAMIDE GELS FOR ELECTROPHORESIS OF INFECTED CELL POLYPEPTIDES

1. Stock Solution Receipes

Solution A	
1N HCl	24.0 ml
Tris	18.1 g
TEMED	0.12 ml
Water to:	100.0 ml

Solution B	
1N HCl	48.0 ml
Tris	5.98 g
TEMED	0.46 ml
Water to:	100.0 ml

Solution 2xC-BIS	
Acrylamide	56.0 g
Bis	5.0 g
Water to:	100.0 ml

Solution 2xC-DATD	
Acrylamide	68.8 g
DATD	1.84 g
Water to:	100.0 ml

Solution D	
Acrylamide	20.0 g
Bis	5.0 g
Water to:	100.0 ml

Solution E	
Riboflavin	4.0 g
Water to:	100.0 ml

Solution F	
Sucrose	40.0 gm
Water to:	100.0 ml

Solution 4xG	
Ammonium persulfate	0.56 g
Water to:	100.9 ml

Tracking Dye	
Bromphenol blue	5.0 g
Water to:	100.0 ml

Coomassie Brilliant Blue	
Coomassie blue	1.25 g
Destaining buffer to:	500.0 ml

Gel Buffer	
10X Tris-Gly	100.0 ml
10% SDS	10.0 ml
Water to:	1000.0 ml

10X Tris-Glycine	
Tris	30 g
Glycine	144 g
Water to:	1000 ml

10% SDS	
SDS	50.0 g
Water to:	500.0 ml

Destaining Buffer	
Methanol	25%
Acetic Acid	7%
Water	68%

2 X SUM	
10% SDS	0.2 ml
5 M Urea	0.2 ml
mercaptoethanon	0.2 ml
Water	0.4 ml

1N HCl	
conc. HCl	8.3 ml
Water to:	100.0 ml

2. Working Solution Receipes

STACKING GEL

Preparative		Slab	
(B)	4.0 ml	(B)	1.0 ml
(D)	4.0 ml	(D)	1.5 ml
(E)	4.0 ml	(E)	1.0 ml
(F)	16.0 ml	(F)	3.5 ml
10% SDS	0.28 ml	10% SDS	0.07 ml

SEPARATING GEL

	Preparative		
	6%; ml	7%; ml	10%; ml
(A)	15.0	15.0	15.0
(2xC)	6.43	7.5	10.68
(H_2O)	30.47	29.4	26.2
(10% SDS)	0.6	0.6	0.6
(4xG)	7.5	7.5	7.5

Slab	
(A)	5.0 ml
(2xC)	2.5 ml
(4xG)	2.5 ml
10% SDS	0.2 ml
Water	9.8 ml

VITA

Steven Allen Sanders was born in Millington, Tennessee, on May 20, 1953. He attended the Millington Central Elementary and High Schools where he participated in baseball, basketball and tennis. He was awarded a high school diploma in June, 1971. The following September he entered Memphis State University and graduated with a Bachelor of Science degree in biology in December, 1975. Following graduation he won a position as research assistant in the influenza laboratories of Virginia S. Hinshaw and Robert G. Webster at St. Jude Children's Research Hospital in Memphis, Tennessee.

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The author is married to Martha F. Wheeler Sanders and has two daughters, Rachel Kelley and Kimberly Erin, born January 4, 1981. He will begin work toward a Doctor of Medicine degree at the University of Tennessee Center for the Health Sciences, Memphis, Tennessee, in August, 1981.